IN THE MATTER OF:

LANCE ARMSTRONG AND TAILWIND SPORTS, INC.	§ § §	BEFORE THE HONORABLE
	§	RICHARD FAULKNER, RICHARD
v.	§	CHERNICK AND TED LYON,
	§	ARBITRATORS
	§	
SCA PROMOTIONS, INC.	§	

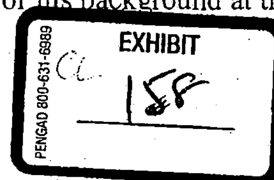
AFFIDAVIT OF DR. CRAIG NICHOLS

Before me this day personally appeared Dr. Craig Nichols, a person to me well known, and upon his oath stated that the following is true and correct and within his personal knowledge:

1. My name is Dr. Craig Nichols. I am currently Professor of Medicine and Chief of Hematology/Oncology at Oregon Health and Sciences University. I have been a doctor since graduating medical school in 1978 and a cancer and blood specialist (hematologist) since 1985. I received my training in Hematology at University of Miami and Hematology/Oncology training at Indiana University. I joined the faculty there and moved through the academic ranks to professor in 1996. In 1998, I was recruited to be the Chair of Hematology/Oncology at Oregon Health and Sciences University. Indiana University is the foremost center in the world for the research and care of patients with testicular cancer and I developed a substantial experience with this disease there. My research on the subject is well known and, I can say, without contradiction, that my reputation as a clinician and researcher in testicular cancer is worldwide.

CANCER TREATMENT

2. On October 19, 1996 the Lance Armstrong made an appointment with me because he had been diagnosed with testicular cancer. I did not know Lance Armstrong or his background at that



time. He had been diagnosed in Austin, Texas under the care of Dr. Dudley Youmans. His diagnosis had been established when he began to cough up blood. Investigation identified numerous rounded abnormalities on his chest X-ray that were highly suspicious for metastasis. Examination in Austin revealed a testicular mass. He underwent tumor marker evaluation and was found to have massively elevated testicular cancer tumor markers (beta human chorionic and gonadotropin). He underwent radical orchiectomy (removal of the testis) and was found to have choriocarcinoma of the testis along with a small amount of malignant teratoma. He received an initial cycle of chemotherapy in Austin and, then developed worsening headaches. Evaluation revealed brain metastases. With the rapidly worsening situation, Lance Armstrong sought expert opinions on the management of the disease and was seen by myself on October 19, 1996. I have reviewed the records of that encounter. At that time, I repeated his blood tumor markers, had the pathology of the resected testis reviewed by the testis cancer pathologist at Indiana University and reviewed the available films personally and along with the radiologists and neurosurgeons at Indiana University. I examined Lance Armstrong and found that he had a surgical resection of his testis. I can say without equivocation that this careful review of the data and repeating of the tumor markers confirmed without a shadow of a doubt that Lance Armstrong was suffering from advanced disseminated choriocarcinoma of the testis. He underwent resection of his brain disease at Indiana about one week later and was found to have some viable cancer. I began treating Lance Armstrong soon after with a second round of chemotherapy with a modified regimen on October 28, 1996. I continued to treat Lance Armstrong until December 13, 1996 and then prescribed a course of follow-up testing.

3. I began treating Lance Armstrong with chemotherapy on October 28, 1996. Lance Armstrong underwent chemotherapy given intravenously for five days every three weeks for three additional cycles. He received an aggressive combination of cisplatin, etoposide and

ifosfamide.

4. Following treatment, I saw Lance Armstrong until October 2001. It is vital to monitor a patient once the testicular cancer has been treated to identify recurrence at an early and treatable time. The usual period for following cancer patients is five years. After the five years the chances of the cancer returning are negligible.

5. Unfortunately, chemotherapy has numerous side-effects. It is usual for Erythropoietin (more commonly known as EPO) to be administered to cancer sufferers to counter certain of these side-effects. As a consequence of the chemotherapy, Lance Armstrong became very anemic, developed a low white blood cell count, and significant nausea and vomiting and, as a result, he was treated with a number of drugs including EPO. As well as EPO Lance Armstrong received ondansetron, lorazepam and dexamethasone for nausea and brain swelling and filgrastim for management of low white blood cell counts.

EPO

6. EPO is the most commonly used drug in the treatment of chemotherapy induced anemia. It is essential to minimize the degree of anemia in cancer patients undergoing chemotherapy to avoid blood transfusions and the profound fatigue associated with chemotherapy-induced anemia. At one point, Lance Armstrong developed a hematocrit of 25gm/dl (normal range for males 42-47). For these reasons, Lance Armstrong was administered with EPO. There is nothing irregular or abnormal about the use of EPO in cancer patients and, indeed, it is one of the most common supportive care drugs given in conjunction with chemotherapy.

7. Lance Armstrong responded positively to the chemotherapy and chemotherapy was stopped on December 13, 1996. The use of EPO on Lance Armstrong was therefore stopped in early January, 1997. At no time after this date did I or, to the best of my knowledge, any of my colleagues administer EPO to Lance Armstrong. There would have been no reason to do so.

MEDICAL FILE

8. While I do not, and cannot, waive Lance Armstrong's medical privilege, I would like to point out that I have, as a result of the preparation of this Affidavit, reviewed Lance Armstrong's medical file. I used the notes contained within to refresh my memory about his treatment.

9. Following successful treatment of his cancer in 1996 I continued to check Lance Armstrong's blood levels on a regular basis from January 1997 to October 2001. Initially, he had regular evaluation every several months for the first year, every four months in the second year and twice yearly to 2001.

10. I can confirm that, during that monitoring period, I saw nothing irregular in Lance Armstrong's hemoglobin or hematocrit levels. Lance Armstrong's blood levels remained consistent and did not fluctuate outside the normal range. I can confirm that at the time of the check-ups, and also upon reviewing the material in the file, there is nothing irregular with Lance Armstrong's red blood cell levels throughout. I am a blood specialist and very familiar with the use and effects of EPO. Had Lance Armstrong been using EPO to enhance his cycling performance, I would have likely identified differences in his blood levels. After all, I had treated him and administered EPO during his treatment years when he was not cycling between October 1996 and January 1997 and was very familiar with his blood levels.

11. I wish to point out also that the "performance-enhancing" effects of EPO last for approximately two weeks following administration. Lance Armstrong was administered EPO between October 1996 and January 1997. I understand that Lance Armstrong only returned to professional cycling in February 1998. Therefore, it is undoubtedly the case that the administration of EPO for the treatment of Lance Armstrong's chemotherapy-induced anemia cannot have had any performance-enhancing effects on Lance Armstrong's cycling. In addition, the fact that throughout the frequent check-ups until October 2001, when they ceased, I did not

notice any unusual or irregular blood cell levels in Lance Armstrong's blood, indicates to me that Lance Armstrong was not administering EPO between January 1997 and October 2001.

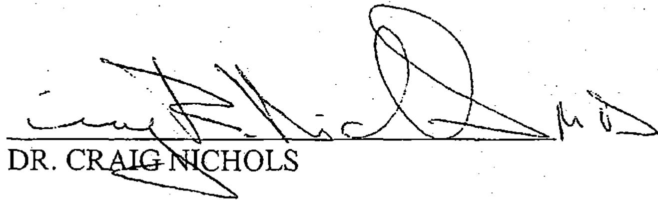
ALLEGED "CONFESSION" OF LANCE ARMSTRONG'S EPO USAGE PRIOR TO CANCER TREATMENT

12. I did not have any knowledge of the background of Lance Armstrong before October 19, 1996. I have been told that it is alleged that Lance Armstrong admitted to his doctors, in front of other non medical personnel, that he had used performance-enhancing drugs prior to being diagnosed with cancer. I have no recollection of being present during any conversation where Lance Armstrong stated this. Though I was not Lance Armstrong's sole physician, I was responsible for the majority of his treatment and would have been present at every large meeting where discussions took place or decisions were made. I have, as mentioned above, had the opportunity to review Lance Armstrong's medical file and can confirm that no entry has been made, neither by me, nor, by any other doctor that saw Lance Armstrong, to the effect that Lance Armstrong had been taking performance-enhancing drugs. I have never seen any evidence, either from myself or any other doctor, that indicates Lance Armstrong admitted, suggested or indicated that he has ever taken performance-enhancing drugs. His medical file from Indiana University Medial Center shows that during his treatment at the Center he was asked questions regarding his medical history over 20 times, which included questions regarding his past medical history and past medications and drugs taken. Nothing in the chart indicates he ever said or responded that he had taken performance-enhancing drugs. The anesthesia and surgical pre-operative notes from October 23, 1996, the day before his brain surgery, are particularly instructive. In that situation, the anesthesiologist and the doctor are visiting with the patient in a very serious situation where accuracy in responses is very important. These doctors disclosed the risks of the brain surgery and anesthesia to Lance, including damage to adjacent tissue, and

nuerological decline such as weakness, numbness, speech and vision problems and they discussed his medical history. Had there been any indication from Armstrong to either of these physicians that he had used performance enhancing drugs, that response would be noted in his records. There is no such note. I and other medical personnel visited with Armstrong about his medical history before his chemotherapy started on October 28, 1996. Lance Armstrong never admitted, suggested or indicated that he has ever taken performance-enhancing drugs. Had this been disclosed to me, I would have recorded it, or been aware of it, as a pertinent aspect of Lance Armstrong's past medical history as I always do, for example, for prior smoking history, alcohol use, illicit drug use and HIV risk factors for each and every patient. Had I been present at any such 'confession,' I would most certainly have vividly recalled the fact. As stated previously, I did not know Lance Armstrong personally or professionally at the time of the first encounter, therefore, there should be no suggestion that I may have somehow purposely omitted to record or recall Lance Armstrong's confession. In any event, I would have recorded such a confession as a matter of form, as indeed, would have my colleagues. None was recorded.

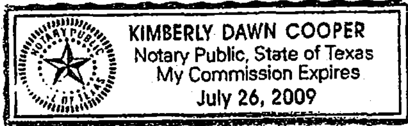
13. Though doctors are under a professional obligation to record all matters regarding a patient's medical history in his/her notes, it would be unusual to ask a professional athlete who has been diagnosed with testicular cancer whether or not he had previously used performance-enhancing drugs. I have treated other athletes with testicular cancer and don't recall ever asking them whether or not they have used performing-enhancing drugs.

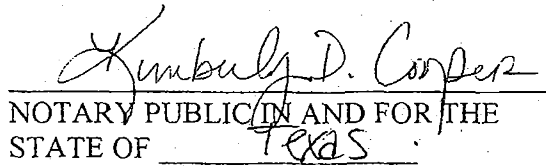
14. While on this point, I believe that it is important to respond to allegations that perhaps the use of performance-enhancing drugs such as EPO can cause cancer and indeed may even have caused cancer in Lance Armstrong. There is no established scientific evidence that EPO can cause testicular cancer. From my treatment of Lance Armstrong I am confident that his cancer could not have, and indeed was not, caused by the use of performance-enhancing drugs.



DR. CRAIG NICHOLS

SUBSCRIBED AND SWORN TO before me this 8 day of December, 2005.





NOTARY PUBLIC IN AND FOR THE
STATE OF Texas

My Commission Expires: 07/09

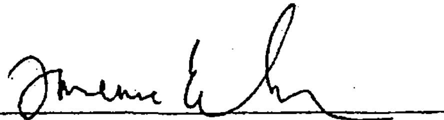
AFFIDAVIT OF MEDICAL RECORDS FROM
INDIANA UNIVERSITY HOSPITAL

BEFORE ME, the undersigned authority, on this day appeared the Affiant, who, being duly sworn, deposed as follows:

"My name is Lawrence Einhorn, I am of sound mind, capable of making this affidavit, and personally acquainted with the facts herein stated:

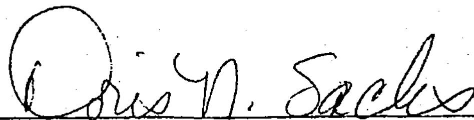
I have custody of a copy the medical records from Indiana University Hospital for Mr. Lance Armstrong (hereinafter "health care provider"). Attached to this affidavit are 279 pages of medical records. The records are the complete set of the Indiana University medical records for Mr. Lance Armstrong from October of 1996 to Present, with the limited exception that the attached copy of these records does not contain copies of the actual x-ray films, scans or other such imaging tests. These records are kept by the health care provider in the regular course of business, and it was the regular business practice of the health care provider for an employee or representative of the health care provider, with knowledge of the act, event, condition, opinion, or diagnosis recorded to make the records or to transmit information thereof to be included in such records to other employees in order to secure accurate diagnosis and/or treatment of the patient. The records were made at or near the time of the act, event, condition, or diagnosis recorded or reasonably soon thereafter.

The records attached hereto are exact duplicates of the original."



Affiant

SUBSCRIBED AND SWORN TO BEFORE ME on this 7th day of December 2005.



NOTARY PUBLIC in and for the State of Indiana

IN THE MATTER OF:

LANCE ARMSTRONG AND TAILWIND SPORTS, INC.	§	
	§	BEFORE THE HONORABLE
v.	§	RICHARD FAULKNER, RICHARD CHERNICK AND TED LYON, ARBITRATORS
	§	
SCA PROMOTIONS, INC.	§	

AFFIDAVIT MARIO ZORZOLI

Before me this day personally appeared Mario Zorzoli, a person to me well known who, upon his oath, stated as follows:

"My name is Mario Zorzoli. I am a resident and citizen of Switzerland; I am over the age of eighteen (18) years, have never been convicted of a criminal offense, and am fully competent to make this Affidavit.

I am the Health Manager of the Union Cycliste Internationale ("UCI"). In this capacity, I am involved with, and intimately familiar with, the testing protocols and results of anti-doping tests and health tests administered to participants and competitors in the Tour de France bicycle competition. I am a medical doctor and I am in charge of the scientific and medical aspects of doping control. I am involved in the scientific and medical aspects of results management of doping tests. I am in charge of the organization, results management and follow-up of health tests.

As a preliminary matter, the anti-doping tests in connection with the Tour de France are performed by the French Ministry of Sports in collaboration with UCI . Testing is conducted pursuant to procedures and protocols prescribed by French anti-doping legislation. The laboratory which performs the analysis of test specimens is the Anti-doping laboratory of Châtenay-Malabry (FRA) and is also "WADA (World Anti-Doping Agency) Accredited" and was accredited by the IOC before.

In 1998, the international cycling world was stunned by a major drug/doping scandal that focused upon Team Festina team but did reveal further abuses in the sport of cycling. This scandal was widely publicized in both Europe and the United States. As a result, and in order to preserve the integrity of UCI events, major improvements and modifications were made to the testing protocols employed at the Tour de France and other competitions. For example, during the 2000 Tour de France, a new testing procedure and methodology for the detection of EPO ("erythropoietin") was being developed and later became available. EPO is a drug which tends to boost hemotocrit levels (essentially, the percentage, by volume, of red blood cells in the blood), thereby increasing endurance. Because the new testing procedures and protocols for EPO were unavailable at the time of the Tour de France race in 2000, all urine test samples were frozen and subjected to the new EPO testing procedures when they were perfected. Thus, had any competitors utilized EPO in conjunction with the 2000 Tour de France, the French authorities would have discovered such use when they tested the frozen urine samples in early 2001. ."

" I have been provided a copy of an allegation in the filings of SCA Promotions, Inc. in which it is alleged, among other things, that the UCI testing processes are ineffective because a) the results analysis processes utilized cannot detect many, if not all, modern Performance Enhancement Drugs ("PED"); b) testing procedures have identifiable weaknesses that undermine their integrity, such as giving riders advance notice; and c) post-testing procedures are highly irregular and suspect. SCA also asserts that many riders are warned of "problems" with test results, then no action is taken by the UCI."

"Those statements, if they are intended to apply to the UCI testing procedures or to testing procedures at the Tour de France, are untrue. I would point out that the Tour de France tests are performed by official doctors appointed by the French Sport Ministry. These doctors may be assisted by a representative of the sports federation, and UCI sends an anti-doping inspector to assist the French official doctor. It is the doctor who is responsible for the testing. As stated before, the laboratory which performs the analysis is the state-owned and "WADA

accredited* laboratory of Châtenay - Malabry (FRA). This laboratory is, as are all other WADA accredited laboratories, entirely independent from UCI and has an excellent reputation. It was this laboratory that developed the EPO-test.

Whereas the French authorities conduct doping tests, UCI at the Tour de France (as at other cycling competitions) also conducts so-called health tests. I have produced a copy of the protocols and procedures employed at the 2004 Tour and have attached them to this affidavit. These health tests are blood tests aiming at identifying atypical blood values. Such values may be caused by various factors, including the use of prohibited substances or methods. If the values are outside the norm laid down in the UCI regulations, the rider will be deemed unfit for cycling and may not participate in races for at least two weeks. (This means that a rider has to leave the race if his blood values are outside the norm, even if the EPO test would be negative). There are reasons for such a policy, not the least of which is that blood values outside the norm, e.g. high haematocrit levels, produce or indicate health risks. If the values are outside the norm or only suspect, the rider will also be submitted to an EPO doping test. The UCI anti-doping inspector will suggest to the official anti-doping doctor in the Tour de France that the rider concerned is specifically tested for EPO. In 2004, numerous urine antidoping tests and blood antidoping tests were administered at the Tour de France. Mr. Armstrong was submitted to both kinds of tests on numerous occasions.

At every stage of the Tour de France, about six riders are tested, including the winner of the stage, two riders selected at random, the current leader (wearing the Tour de France yellow jersey) and any riders selected on the basis of blood testing. At time trial events, the first three finishing riders and two additional at random selected riders are tested.

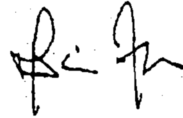
There is no advance notice for health tests other than the time necessary to check in at the testing room (maximum ten minutes). There is no advance notice for doping tests other than the knowledge that the leader of the race and the winner of the stage will be tested. Riders selected at random by the French doctor are informed some minutes before finishing the race so that they know that they have to remain available for testing after the finish.

The reports of the laboratory with the analysis results are sent to another French independent body, the CPLD (Conseil de Lutte et de Prevention du Dopage), according to the French legislation. This official body of French public law is in charge of results management and of disciplinary proceedings against foreign riders (the French Cycling Federation is in charge of disciplinary proceedings against its own licensees). Copies of the laboratory reports are also sent to the French Ministry of Sports, WADA and UCI. It is unthinkable that no action would be undertaken in the case of a laboratory report identifying the presence of a prohibited substance or method.

"In addition to the race-related tests, out-of-competition tests are administered by the UCI and various other anti-doping agencies without warning."

"I confirm that, in the 2004 Tour de France, Mr. Lance Armstrong was tested several times (8 antidoping urine tests / 1 antidoping blood test / 2 health blood tests) in conformity with the French legal protocols and procedures and that all of those Laboratory results were negative. I also confirm that Mr. Armstrong has been tested numerous additional times at the Tour de France in prior years and has never violated any of the rules and regulations of the race or tested positive for any banned or illegal substance or procedure. I also confirm that Mr. Armstrong never showed atypical blood values".

Further affiant sayeth not.



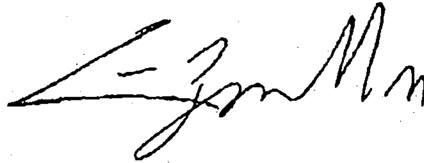
MARIO ZORZOLI

SUBSCRIBED AND SWORN TO before me this _____ day of May, 2005.

NOTARY PUBLIC

Légalisation numéro 7'427 : ———————————————————

Sur la base d'une comparaison de signature, le soussigné, Charles-Pascal GHIRINGHELLI, notaire à Aigle pour le canton de Vaud, atteste l'authenticité de la signature apposée ci-dessus par Monsieur Mario Zorzoli, domicilié à Genève. —— Aigle, le deux mai deux mille cinq. ———————————————



January 2003

Procedure for the UCI medical inspectors
in the context of blood tests

Preparation

The Sports Safety and Conditions Commission (SSCC) designates two medical inspectors. One is designated as 'mission leader' and the other one as 'assistant inspector'. It is the 'mission leader' who is responsible for the mission.

The medical inspectors (hereafter: the inspectors) receive instructions from the SSCC about the hotel or hotels where the blood tests will take place. They may be sent to the location two days before the date of the test, in order to inspect the premises and the team hotels. The inspectors may be given the task of booking premises for the test, they will be responsible for paying for these premises and will then be reimbursed on presentation of a simple expenses slip accompanied by a receipt. Preferably, two rooms of hotel rooms will be booked (or alternatively, the Team Manager's or team doctor's room), and unsuitable premises must not be accepted, i.e.: basements, cellars, corridors or 'open' rooms that allow 'outside' people to look in.

On the eve of the test, and in the place indicated by the SSCC (mission hotel), the 'mission leader' must contact the Independent Institute (laboratory) designated by the SSCC to conduct the blood tests (hereafter: the institute), to prepare the organisation of the test. He ensures that the material and equipment are installed on the eve of the test. He fills in the 'Notice to the riders' and 'Blood test' forms with the information he already has at his disposal.

The inspectors receive instructions from the SSCC regarding the riders or teams to be tested, as well as information about the hotels where the riders to be tested are staying. The inspectors must contact the president of the commissaires panel on the eve of the test to check whether any changes have been made in this regard.

The 'mission leader' draws up a list of the riders to be examined. If lots are to be drawn, the 'mission leader' conducts this on the eve of the test, in the presence of the representative of the institute.

Organisation of the test

The 'mission leader' must plan the timetable of the blood tests (in co-operation with the assistant inspector and the institute) so that the blood tests are finished three hours before the start of the race and before 9 a.m.

The SSCC wishes to give priority to the QUALITY of the tests rather than the QUANTITY. This means that sometimes, the SSCC may decide to test only 2 or 3 teams, or only the most distant teams.

If several teams are staying in the same hotel, it is wise to test some riders per team, but from ALL the teams.

The inspectors give the notice form personally to the team manager or the team leader or to another team representative. They ask for the form to be signed as an acknowledgement of receipt, and take it back. One copy is given to the team representative. If the latter does not

CL 0305

The inspectors give the notice form personally to the team manager or the team leader or to another team representative. They ask for the form to be signed as an acknowledgement of receipt, and take it back. One copy is given to the team representative. If the latter does not sign it to acknowledge receipt, the inspectors mention this on the form. If there is no team representative, or if he cannot be found, the inspectors try to reach the riders individually and give them the copy of the form directly. They get each rider to sign the original.

Notification takes place at most ten minutes before the time when the riders concerned must present themselves at the test premises. The inspectors will make sure to remind them at the time of notification that all the riders of the same team must present themselves at the same time at the test premises.

Conduct of the test

The fact that a rider does not have an empty stomach for a test does not prevent the blood test and does not falsify the analysis.

It is no longer necessary for the inspectors to take the riders' licence.

The riders have the option of waiting 10/15 minutes between presenting themselves, which happens just 10 minutes after they have been notified, and the blood sample being taken. During this interval, they must without fail be monitored by the medical inspector.

The inspector (the inspector who is on the test premises) fills in the 'Blood test' form while the institute proceeds with taking the blood samples. He makes sure that the form contains all the required information before it is signed by the rider, his attendant if there is one, by the representative of the institute and by himself. If the rider and/or his attendant do not sign, the inspector mentions this on the form and indicates the reason.

Procedure if the limit value is exceeded

Before calling a rider for a counter-analysis, make sure that the institute has checked the Hct value of the A sample with the centrifuge and that the result is confirmed.

If the result of the analysis of the A sample shows a haematocrit value exceeding the maximum level, the 'mission leader' informs the rider concerned or his representative about this while on the test premises. If the rider or his representative are no longer on the premises, the B sample will not be analysed and the result of the analysis of the A sample is final. If the rider or his representative are present, he can be present at the analysis of the B sample. The representative must be in possession of a written authorisation.

The 'mission leader' must fill in the 'Declaration of Incapability' form in the following cases :

- the rider did not present himself for the blood test within the time limit
- the rider presented himself but refused the test
- the examination indicated a haematocrit level in excess of the maximum level.

He must draw up a form in four copies for each rider concerned:

- one for the rider
- one for the rider's national federation
- one for the commissaires panel
- one for the file to be forwarded to the SSCC

The copy for the rider is given to his team manager or team leader. If there is no team representative, the 'mission leader' will forward the form to the riders concerned in a way he chooses.

The rider is declared unfit for a minimum period of 15 days. The 'mission leader' informs the SSCC, who will invite the rider to undergo another blood test, at the earliest 15 days after the first test, in order to determine whether the rider can resume his sports activities.

The latter must go to an independent institute accredited by the UCI for the performance of the blood tests. However, the rider and/or his representative are free to choose the laboratory. The UCI reserves the right, however, to impose the laboratory.

CL 0307

Avril 2004

Protocole de mesure des paramètres sanguins avec l'analyseur Sysmex de la série XT-2000I

A. INSTALLATION, CALIBRATION ET CONTRÔLE:

- Installer l'appareil et le PC selon les modalités habituelles. Passer les contrôles Low, Medium et High 2 fois et vérifier s'ils sont dans l'intervalle défini par le constructeur.
- Si les contrôles ne sont pas corrects alors effectuer un autorinse, un clog remove et éventuellement un shutdown. Une fois le shutdown effectué, attendre environ 40 minutes afin que la javel agisse.
- Repasser les contrôles et vérifier s'ils sont dans l'intervalle défini par le constructeur.
- Prélever un sang frais, l'homogénéiser pendant 15 minutes et l'analyser avec le Sysmex.
- Vérifier la valeur d'hématocrite avec la centrifugeuse. La valeur de l'hémoglobine doit correspondre à Hct / 3 environ.
- Ce même sang doit être analysé 7 fois et le coefficient de variabilité de l'hématocrite et de l'hémoglobine doit être inférieur a 1.8 % et celui des reticulocytes (%) doit être inférieur a 15 %. Si les valeurs sont dans l'intervalle défini par le constructeur, alors effectuer un « Shutdown».

B. PRISE DE SANG – ECHANTILLONS A ET B

- Le coureur doit être en position assise.
- Le garrot ne doit pas être posé trop longuement inutilement (délai de moins de une minute entre la pose du garrot et l'apparition du sang dans le tube).
- Deux tubes de 2,7 ml de sang sont prélevés par une ponction unique. Ils sont désignés arbitrairement échantillons A et B. Ils sont étiquetés avec un numéro identique.
- Le tube A est roulé au minimum 15 minutes et analysé immédiatement tel que la procédure le décrit ci-dessous dans le point C.
- Le tube B est placé dans un flacon numéroté (Versapack). Le flacon est attribué à l'équipe toute entière. Le numéro du flacon est inscrit sur le formulaire "Contrôle sanguin" sous le point 9 et sera sellé par le manager de l'équipe dès que tous les échantillons B auront été introduits.
- Cet échantillon sera utilisé pour la contre-analyse.
- Si pour une raison quelconque, le remplissage du deuxième tube présente des difficultés par la même ponction, il sera demandé au coureur s'il désire une deuxième ponction, sinon il admet qu'en cas de résultat entraînant une déclaration d'inaptitude pour l'échantillon A, la deuxième analyse se fera également sur l'échantillon A. Cette condition est acceptée sous le chiffre 11 du formulaire "Contrôle sanguin".
- En cas de résultat entraînant une déclaration d'inaptitude pour l'échantillon A, le coureur peut demander que l'échantillon B soit ouvert devant lui et analysé suivant les mêmes règles que pour l'échantillon A et à l'aide d'une centrifugeuse. Après une contre-expertise, c'est le résultat de l'échantillon B qui sera pris en compte de manière définitive. La demande d'ouverture de l'échantillon B doit être formulée dans un temps raisonnable, après l'annonce du résultat de l'échantillon A, en tenant compte des impératifs de

CL 0308

la course et de la qualité des analyses. Ce délai sera discuté le cas échéant entre l'inspecteur médical, le directeur sportif du coureur et le responsable scientifique de l'équipe médical (cf. art 13.1.055 du règlement UCI).

C. ANALYSE DE L'ECHANTILLON A

- *Préparation des sangs*
  - Les sangs sont roulés pendant 15 minutes au minimum avant l'analyse pour homogénéisation et stabilisation de la température.
  - Effectuer un autorinse avant toute analyse
- *Mesures*
  - Effectuer une seule mesure sur l'échantillon A si Hct ≤ 50% ou Hb ≤ 17 g/dl ET Réti ≥ 0.4%
  - Le coureur est déclaré apte.

a. Hématocrite > à 50% ET hémoglobine > à 17 g/dl

- Effectuer une deuxième mesure sur l'échantillon A
- Si Hct ≤ à 50% ou Hb ≤ 17 g/dl, le coureur est déclaré apte.
- Si Hct > à 50% et Hb ≤ 17 g/dl alors écrire la phrase suivante à côté du résultat Hct : « Résultat non validé par d'autres paramètres hématologiques »
- Si la deuxième mesure montre Hct > à 50% ET hémoglobine > à 17 g/dl alors il y a contre-expertise sur l'échantillon B.

AVANT D'APPELER LE COUREUR POUR LA CONTRE-EXPERTISE SUR L'ECHANTILLON B, MESURER L'HCT DE L'ECHANTILLON A AVEC LA CENTRIFUGEUSE

b. Réti% < 0.4

- Effectuer une deuxième mesure sur l'échantillon A
- Si Réti% < 0.4, calculer l'index de stimulation OFF avec les Hb mesurées selon la formule :

$$\text{Index de stimulation OFF} = Hb * 10 - 60 \sqrt{\text{Réti}}$$

- Pour les coureurs qui possèdent un Certificat d'Hct naturellement élevé, dont les valeurs d'Hb mesurées sont supérieures à 17 g/dl, on considérera pour le calcul de l'index la valeur de 17 g/dl (16 g/dl pour les femmes)
- Si les deux index sont > à 133 (123 pour les femmes), alors il y a contre-expertise sur l'échantillon B

D. ANALYSE DE L'ECHANTILLON B

Le coureur ou son mandataire est informé qu'il peut assister, dans un délai défini par l'inspecteur médical, à l'analyse de l'échantillon B.

CL 0309

Avant de procéder à la contre-expertise, passer 2 fois le contrôle High , 2 fois le contrôle Medium et 2 fois le contrôle Low : les valeurs doivent se situer dans l'intervalle défini par le fabricant des contrôles. Pas toujours possible !!!

La contre-expertise sera effectuée à l'aide de l'analyseur Sysmex de la série XT-2000i et d'une centrifugeuse.

a. *Préparation des sangs*
   - Le tube de sang EDTA est roulé pendant 15 minutes au minimum avant l'analyse pour homogénéisation et stabilisation de température.
   - 10 minutes avant l'analyse de l'échantillon B de la contre expertise, sortir l'appareil en «stand by» en pressant sur la touche « start », puis effectuer un « autorinse ».

b. *Analyse de l'échantillon B pour hématocrite > 50%*
   - Effectuer une mesure sur l'échantillon B. Si Hct ≤ 50% (47%) ou Hb ≤ 17 g/dl (16 g/dl), le coureur est déclaré apte.
     Dans le cas contraire, procéder à une deuxième mesure.
   - Lors de la deuxième mesure, si l'hématocrite est > à 50 % ET l'hémoglobine > à 17g/dl, alors effectuer une centrifugation.
   - Si la valeur hématocrite déterminée par la centrifugeuse est > à 50%, le coureur est déclaré inapte.
   - Dans tous les cas la valeur la plus basse sera la valeur retenue.
   - Si Hct > à 50% et Hb ≤ 17 g/dl alors écrire la phrase suivante à côté du résultat Hct : « Résultat non validé par d'autres paramètres hématologiques »
   - Les valeurs pour les femmes sont respectivement 47% pour l'hématocrite et 16 g/dl pour l'hémoglobine.

c. *Analyse de l'échantillon B pour index off > 133*
   - Effectuer une mesure sur l'échantillon B. Si l'index off calculé est ≤ 133, le coureur est déclaré apte. Dans le cas contraire, procéder à une deuxième mesure de l'échantillon B.
   - Lors de la deuxième mesure, si l'index off calculé est > 133, alors le coureur est déclaré inapte.
   - Dans tous les cas la valeur la plus basse est la valeur retenue.
   - La valeur de l'index OFF pour les femmes est de 123

CL 0310

IN THE MATTER OF:

LANCE ARMSTRONG AND TAILWIND SPORTS, INC.	§ §	
	§ § § §	BEFORE THE HONORABLE RICHARD FAULKNER, RICHARD CHERNICK AND TED LYON, ARBITRATORS
v.		
SCA PROMOTIONS, INC.	§ §	

AFFIDAVIT OF CHRISTIAN VARIN

Before me this day personally appeared Christian Varin, a person to me well known who, upon his oath, stated as follows:

"My name is Christian Varin. I am a resident and citizen of Switzerland; I am over the age of eighteen (18) years, have never been convicted of a criminal offense, and am fully competent to make this Affidavit.

I am the Anti-doping Manager of the Union Cycliste Internationale ("UCI"). In this capacity, I am involved with, and intimately familiar with, the testing protocols and results of antidoping tests administered to participants and competitors in the Tour de France bicycle competition. As a preliminary matter, the anti-doping tests in connection with the Tour de France are performed by the French Ministry of Sports in collaboration with UCI. Testing is conducted pursuant to procedures and protocols prescribed by French anti-doping legislation. The laboratory which performs the analysis of test specimens is the Anti-doping laboratory of Châtenay-Malabry (FRA) and is also "WADA (World Anti-Doping Agency) Accredited" and was accredited by the IOC before.

In 1998, the international cycling world was stunned by a major drug/doping scandal that focused upon Team Festina team but did reveal further abuses in the sport of cycling. This scandal was widely publicized in both Europe and the United States. As a result, and in order to preserve the integrity of UCI events, major improvements and modifications were made to the

testing protocols employed at the Tour de France and other competitions. For example, during the 2000 Tour de France, a new testing procedure and methodology for the detection of EPO ("erythropoietin") was being developed and later became available. EPO is a drug which tends to boost hemotocrit levels (essentially, the percentage, by volume, of red blood cells in the blood), thereby increasing endurance. Because the new testing procedures and protocols for EPO were unavailable at the time of the Tour de France race in 2000, all urine test samples were frozen and subjected to the new EPO testing procedures when they were perfected. Thus, had any competitors utilized EPO in conjunction with the 2000 Tour de France, the French authorities would have discovered such use when they tested the frozen urine samples in early 2001. ."

" I have been provided a copy of an allegation in the filings of SCA Promotions, Inc. in which it is alleged, among other things, that: the UCI testing processes are ineffective because a) the results analysis processes utilized cannot detect many, if not all, modern Performance Enhancement Drugs ("PED"); b) testing procedures have identifiable weaknesses that undermine their integrity, such as giving riders advance notice; and c) post-testing procedures are highly irregular and suspect. SCA also asserts that many riders are warned of "problems" with test results, then no action is taken by the UCI."

"Those statements, if they are intended to apply to the UCI testing procedures or to testing procedures at the Tour de France, are untrue. I would point out that the Tour de France tests are performed by official doctors appointed by the French Sport Ministry. These doctors may be assisted by a representative of the sports federation, and UCI sends an anti-doping inspector to assist the French official doctor. It is the doctor who is responsible for the testing. As stated before, the laboratory which performs the analysis is the state-owned and "WADA accredited" laboratory of Châtenay - Malabry (FRA). This laboratory is, as are all other WADA accredited laboratories, entirely independent from UCI and has an excellent reputation. It was this laboratory that developed the EPO-test.

Whereas the French authorities conduct doping tests, UCI at the Tour de France (as at other cycling competitions) also conducts so-called health tests. These are blood tests aiming at identifying atypical blood values. Such values may be caused by various factors, including the use of prohibited substances or methods. If the values are outside the norm laid down in the UCI regulations, the rider will be deemed unfit for cycling and may not participate in races for at least two weeks. (This means that a rider has to leave the race if his blood values are outside the norm, even if the EPO test would be negative). There are reasons for such a policy, not the least of which is that blood values outside the norm, e.g. high haematocrit levels, produce or indicate health risks If the values are outside the norm or only suspect, the rider will also be submitted to an EPO doping test. The UCI anti-doping inspector will suggest to the official anti-doping doctor in the Tour de France that the rider concerned is specifically tested for EPO. In 2004, numerous urine antidoping tests and blood antidoping tests were administered at the Tour de France. Mr. Armstrong was submitted to both kinds of tests on numerous occasions.

At every stage of the Tour de France, about six riders are tested, including the winner of the stage, two riders selected at random, the current leader (wearing the Tour de France yellow jersey) and any riders selected on the basis of blood testing. At time trial events, the first three finishing riders and two additional at random selected riders are tested.

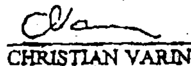
There is no advance notice for health tests other than the time necessary to check in at the testing room (maximum ten minutes). There is no advance notice for doping tests other than the knowledge that the leader of the race and the winner of the stage will be tested. Riders selected at random by the French doctor are informed some minutes before finishing the race so that they know that they have to remain available for testing after the finish.

The reports of the laboratory with the analysis results are sent to another French independent body, the CPLD (Conseil de Lutte et de Prevention du Dopage), according to the French legislation. This official body of French public law is in charge of results management and of disciplinary proceedings against foreign riders (the French Cycling Federation is in charge of disciplinary proceedings against its own licensees). Copies of the laboratory reports are also sent to the French Ministry of Sports, WADA and UCI. It is unthinkable that no action would be undertaken in the case of a laboratory report identifying the presence of a prohibited substance or method.

"In addition to the race-related tests, out-of-competition tests are administered by the UCI and various other anti-doping agencies without warning. "

"I confirm that, in the 2004 Tour de France, Mr. Lance Armstrong was tested several times (8 antidoping urine tests / 1 antidoping blood test / 2 health blood tests) in conformity with the French legal protocols and procedures and that all of those Laboratory results were negative. I also confirm that Mr. Armstrong has been tested numerous additional times at the Tour de France in prior years and has never violated any of the rules and regulations of the race or tested positive for any banned or illegal substance or procedure. I also confirm that Mr. Armstrong never showed atypical blood values".

Further affiant sayeth not.



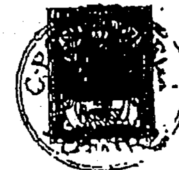
CHRISTIAN VARIN

SUBSCRIBED AND SWORN TO before me this _____ day of May, 2005.

NOTARY PUBLIC

Légalisation au dos

Légalisation numéro 7'428: ——————————
Le soussigné, Charles-Pascal GHIRINGHELLI, notaire à Aigle pour le canton de Vaud, atteste l'authenticité de la signature apposée au recto par Monsieur Christian Varin suivant présentation de sa carte d'identité suisse numéro 003147897. ————
Aigle, le deux mai deux mille cinq. ——————————



CL 0243

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IN THE MATTER OF:

LANCE ARMSTRONG AND TAILWIND SPORTS, INC.	§ § §	BEFORE THE HONORABLE RICHARD FAULKNER, RICHARD CHERNICK AND TED LYON, ARBITRATORS
v.	§ § §	
SCA PROMOTIONS, INC.	§ §	

AFFIDAVIT OF LÉON SCHATTENBERG

Before me this day personally appeared Léon Schattenberg, a person to me well known who, upon his oath, stated as follows:

"My name is Léon Schattenberg. I am a resident and citizen of the Netherlands; I am over the age of eighteen (18) years, have never been convicted of a criminal offense, and am fully competent to make this Affidavit.

I am the President of the Anti-doping Commission of the Union Cycliste Internationale ("UCI"). In this capacity, I am involved with, and intimately familiar with, the testing protocols and results of anti-doping tests administered to participants and competitors in the Tour de France bicycle competition. I am involved with defining the anti-doping policy of the UCI and supervising its implementation. I was responsible for setting up the health testing programme which I introduced in 1997. I am informed of all testing results. I am a medical doctor with a years experience in the field of doping. I am responsible for setting up the collaboration with the French authorities concerning doping control at the Tour de France and I am supervising such controls on behalf of UCI, including on the field.

As a preliminary matter, the anti-doping tests in connection with the Tour de France are performed by the French Ministry of Sports in collaboration with UCI . Testing is conducted pursuant to procedures and protocols prescribed by French anti-doping legislation. The laboratory which performs the analysis of test specimens is the Anti-doping laboratory of Châtenay-Malabry

(FRA) and is also "WADA (World Anti-Doping Agency) Accredited" and was accredited by the IOC before.

In 1998, the international cycling world was stunned by a major drug/doping scandal that focused upon Team Festina team but did reveal further abuses in the sport of cycling. This scandal was widely publicized in both Europe and the United States. As a result, and in order to preserve the integrity of UCI events, major improvements and modifications were made to the testing protocols employed at the Tour de France and other competitions. For example, during the 2000 Tour de France, a new testing procedure and methodology for the detection of EPO ("erythropoietin") was being developed and later became available. EPO is a drug which tends to boost hematocrit levels (essentially, the percentage, by volume, of red blood cells in the blood), thereby increasing endurance. Because the new testing procedures and protocols for EPO were unavailable at the time of the Tour de France race in 2000, all urine test samples were frozen and subjected to the new EPO testing procedures when they were perfected. Thus, had any competitors utilized EPO in conjunction with the 2000 Tour de France, the French authorities would have discovered such use when they tested the frozen urine samples in early 2001. ."

" I have been provided a copy of an allegation in the filings of SCA Promotions, Inc. in which it is alleged, among other things, that the UCI testing processes are ineffective because a) the results analysis processes utilized cannot detect many, if not all, modern Performance Enhancement Drugs ("PED"); b) testing procedures have identifiable weaknesses that undermine their integrity, such as giving riders advance notice; and c) post-testing procedures are highly irregular and suspect. SCA also asserts that many riders are warned of "problems" with test results, then no action is taken by the UCI."

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At every stage of the Tour de France, about six riders are tested, including the winner of the stage, two riders selected at random, the current leader (wearing the Tour de France yellow jersey) and any riders selected on the basis of blood testing. At time trial events, the first three finishing riders and two additional at random selected riders are tested.

There is no advance notice for health tests other than the time necessary to check in at the testing room (maximum ten minutes). There is no advance notice for doping tests other then the knowledge that the leader of the race and the winner of the stage will be tested. Riders selected at

CL 0298

random by the French doctor are informed some minutes before finishing the race so that they know that they have to remain available for testing after the finish.

The reports of the laboratory with the analysis results are sent to another French independent body, the CPLD (Conseil de Lutte et de Prevention du Dopage), according to the French legislation. This official body of French public law is in charge of results management and of disciplinary proceedings against foreign riders (the French Cycling Federation is in charge of disciplinary proceedings against its own licensees). Copies of the laboratory reports are also sent to the French Ministry of Sports, WADA and UCI. It is unthinkable that no action would be undertaken in the case of a laboratory report identifying the presence of a prohibited substance or method.

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Further affiant sayeth not.

LEON SCHATTENBERG

SUBSCRIBED AND SWORN TO before me this _____ day of May, 2005.

NOTARY PUBLIC

CL 0299

IN THE MATTER OF:

LANCE ARMSTRONG AND TAILWIND SPORTS, INC.	§ §	
	§ §	BEFORE THE HONORABLE RICHARD FAULKNER, RICHARD
v.	§ §	CHERNICK AND TED LYON, ARBITRATORS
SCA PROMOTIONS, INC.	§ §	

AFFIDAVIT OF TRAVIS T. TYGART

Before me this day personally appeared Travis T. Tygart, who identified himself to me and, upon his oath, swore to the following:

"My name is Travis T. Tygart. I am a resident and citizen of the United States; I am over the age of eighteen (18) years, have never been convicted of a criminal offense, and am fully competent to make this Affidavit. The facts stated in this affidavit are true and correct and within my personal knowledge.

I am Senior Managing Director, General Counsel for the United States Anti-Doping Agency ("USADA"). In this capacity, I am involved with, and intimately familiar with, the drug testing protocols and results management of anti-doping tests administered by USADA.

USADA is an independent, nongovernmental anti-doping agency for Olympic and Paralympic sports in the United States. USADA is a nonprofit Colorado corporation with its office in Colorado Springs, Colorado. USADA was created as the result of recommendations made by the United States Olympic Committee's ("USOC") Select Task Force on Externalization to uphold the Olympic ideal of fair and ethical competition and to represent the interests of Olympic, Pan American, and Paralympic athletes.

USADA is not subject to the control of the USOC but has contracted with the USOC to administer its entire anti-doping program. The USOC has given USADA full authority to execute a comprehensive national anti-doping program encompassing testing, adjudication, education, and research, and to develop programs, policies, and procedures in each of these areas.

USADA began operations October 1, 2000. Its board of directors consists of nine members, five of whom are independent; two of whom are recommended by the Athlete Advisory Council; and, two of whom are recommended by the National Governing Body Council. USADA's professional staff is responsible for managing and coordinating the agency's day to day operations.

All athletes in U.S. Olympic sports, including athlete members of U.S. national governing bodies such as USA Cycling, are subject to the programs of USADA. Because USA Cycling is the U.S. national federation of the Union Cycliste Internationale ("UCI"), the sport of cycling's international sanctioning body, all U.S. cyclists including professional cyclists are subject to testing by USADA. To be recognized as a national federation by the UCI and the USOC, USA Cycling is legally required to follow the protocols of USADA. A true and correct copy of the USASDA Protocol for Olympic Movement Testing is attached hereto as Exhibit A. USADA performs out-of-competition (OOC) testing on elite USA Cycling athletes and also performs testing at USA Cycling-sanctioned events, such as the Tour of Georgia.

By being a licensed member of USA Cycling, like all licensed members, Mr. Lance Armstrong ("Mr. Armstrong") has an obligation to participate in the drug testing programs of USADA. Further, since Mr. Armstrong competes internationally and is an

elite U.S. cyclist, he is in the USA Cycling/USADA OOC drug testing pool. As part of Mr. Armstrong's obligation for being in the OOC testing pool, he must submit to USADA his location information of where he can be located for testing at anytime and anywhere, 24-hours a day, 7-days a week, 365 days a year, with no advance notice of the test. The USADA system of anywhere, anytime no advance notice testing builds in three unavailable attempts or missed tests in an 18 month period before the athlete is subject to a potential sanction for not being at the location for testing. This OOC testing system is one of the strictest in the world by requiring athletes to be available anywhere and anytime for no advance notice testing.

The selection of athletes by USADA for OOC testing is done on a weighted computerized system based in part on international standards. Each sport under USADA's jurisdiction is broken into high, medium, or low risk. Based on this and given USADA's resources, a certain number of tests are allocated to that sport based on these factors. The higher the risk a sport is, the more likely that athlete will be selected for OOC testing. USADA also has the authority to target test athletes.

If an athlete's name is selected for OOC testing, there are steps for the USADA Doping Control Officer ("DCO") to follow in obtaining the sample from the athlete. Attached as Exhibit B to this Affidavit is a true and correct copy of the instructions for a DCO to follow in locating the selected athlete for OOC testing.

USADA also conducts testing at USA Cycling sanctioned events. USADA generally follows the selection criteria for competition testing set forth by UCI but has authority to make its own selections. Generally, the top finishers and additional random selections out of the entire field of athletes are the athletes selected for testing.

When a sample is taken from an athlete, it is sent to an independent World Anti-doping Agency ("WADA") accredited laboratory. If the laboratory reports an adverse analytical result (positive test), USADA notifies the athlete, the USOC, and the sport's national governing body such as USA Cycling. The athlete can then request that his or her "B" sample be tested. If the "B" sample is also positive, then an independent review board reviews the documentary evidence and recommends whether sufficient evidence exists to proceed to a doping hearing. After receiving the review board's recommendation, USADA decides whether or not to charge the athlete with a doping violation. If USADA charges an athlete, the athlete can contest the charge through arbitration before a panel of American Arbitration Association ("AAA") and the Court of Arbitration for Sport ("CAS") arbitrators.

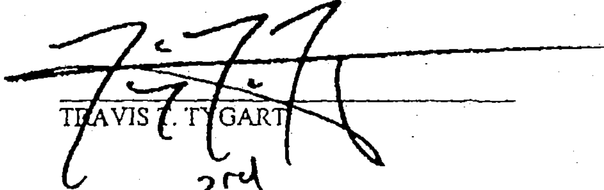
USADA also has the ability to proceed against an athlete for committing a doping violation not involving a positive test. In the event an athlete refuses to test, distributes a prohibited drug or is unavailable for testing, USADA would charge the athlete with a doping violation through the same process described previously.

Athletes that are subject to USADA's jurisdiction are subject to one of the most stringent anti-doping programs in the sports world.

I have reviewed USADA's files regarding Mr. Armstrong. USADA has drug tested Mr. Armstrong twelve (12) separate times on the following dates: November 20, 2001, December 6, 2001, October 22, 2002, November 18, 2003, April 22, 2004, April 23, 2004, April 24, 2004, April 25, 2004, December 7, 2004, January 26, 2005, February 19, 2005, and April 5, 2005. Mr. Armstrong has never had an adverse analytical finding

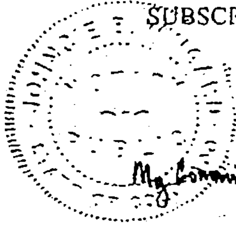
reported to USADA. USADA has never charged Mr. Armstrong with a doping violation for a positive test or being unavailable for testing or otherwise.

Further affiant sayeth not.



TRAVIS T. TYGART

SUBSCRIBED AND SWORN TO before me this 3rd day of May, 2005.



NOTARY PUBLIC

My Commission Expires: 7/5/08

IN THE MATTER OF:

LANCE ARMSTRONG AND TAILWIND SPORTS, INC.	§ § §	BEFORE THE HONORABLE
	§ §	RICHARD FAULKNER, RICHARD CHERNICK AND TED LYON,
v.	§	ARBITRATORS
	§	
SCA PROMOTIONS, INC.	§	

STATE OF CALIFORNIA	§
	§
COUNTY OF LOS ANGELES	§

AFFIDAVIT OF DON CATLIN

Before me this day personally appeared DON CATLIN, a person to me well known, who, upon his oath, stated as follows:

"My name is DON CATLIN. I am employed at the University of California at Los Angeles ("UCLA") and am responsible for the management and operation of the Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology at UCLA. I am over the age of eighteen years and am fully competent to make this affidavit. I have personal knowledge of the statements set forth herein and such statements are true and correct.

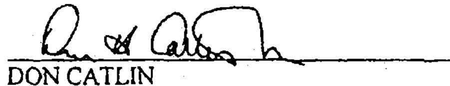
I played a significant role in the institution of athletic drug testing in the United States some 25 years ago. The laboratory for which I have responsibility tests specimens provided by several sporting associations including, but not limited to, the United States Olympic Committee, the National Football League and the NCAA. The specimens which are received at our laboratory are unidentified and tested according to protocols which are developed independently by our laboratory designed to detect a wide range of banned substances, the profiles and coordinates of which are electronically stored in our computers. Thus, if any sample reveals any

banned substance in our database, it will be detected. This database is constantly updated as new or "designer" substances are made known to us.

Our laboratory is the exclusive destination and analytical facility for all specimens taken by the United States Anti Doping Association ("USADA"). While I have no idea whether Mr. Lance Armstrong's specimens have been tested by our lab, as all competitors are anonymous, if a specimen has been collected by USADA from Mr. Armstrong, our lab would have performed the analysis. The protocols and procedures employed by our laboratory are extremely sophisticated and highly reliable. If a USADA specimen was analyzed by our lab without a banned substance being detected, I can state with confidence that such a banned substance was not in detectable amounts in the specimen.

I have attached to this affidavit two papers descriptive of the tests employed to detect EPO ("erythropoietin"), a drug which tends to boost the hemotocrit levels (essentially, the percentage, by volume, of red blood cells in the blood), thereby increasing endurance. This procedure reliably detects EPO if it is present. We also have vast steroid coordinates in our database and the testing procedures and protocols are likewise dependable for the detection of all such substances of which we are aware.

Further Affiant sayeth not.



DON CATLIN

SUBSCRIBED AND SWORN TO before me this ______ day of May, 2005.

NOTARY PUBLIC IN AND FOR THE
STATE OF CALIFORNIA

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Detection of Recombinant Human Erythropoietin in Urine by Isoelectric Focusing

Andreas Breidbach,[1] Don H. Catlin,[1,3*] Gary A. Green,[1,2] Inna Tregub,[1] Henry Truong,[1] and Jeffrey Gorzek[1]

Background: Doping with erythropoietic proteins such as recombinant human erythropoietin (rHuEPO) and darbepoetin alfa is a serious issue in sport. There is little information on the time course of detection of rHuEPO in urine and on methods to evaluate electrophoresis-based data.

Methods: We used a recently described isoelectric focusing method for detecting rHuEPO and endogenous EPO in urine obtained from individuals treated with placebo or epoetin alfa. The latter was administered subcutaneously at 50 IU/kg on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. Blood and urine samples were collected during the morning of study days −3, 0, 2, 4, 7, 9, 11, 14, 16, and 18 and on days 2, 3, 4, and 7 postadministration. We developed visual and numerical (two-band ratio) techniques to evaluate the electropherograms for the presence of rHuEPO.

Results: Compared with the placebo group, the epoetin alfa-treated group responded with increases in hematocrit, reticulocytes, macrocytes, serum EPO, and serum soluble transferrin receptor. The electropherograms showed that the pattern of bands arising from urinary rHuEPO is different from that of endogenous urinary EPO. Both the two-band ratio and the visual technique detected rHuEPO in all 14 epoetin alfa-treated individuals 3 days after the last dose. On the 7th day after the last dose, both techniques detected rHuEPO in approximately one-half of the participants. rHuEPO was not detected in the placebo-treated individuals.

Conclusions: The isoelectric focusing method detects rHuEPO in most urine samples collected 3 days after nine doses of epoetin alfa. The numerical two-band ratio was equivalent to a visual method for detecting rHuEPO in urine.

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Recombinant human erythropoietin (rHuEPO)[4] is a glycoprotein with 165 amino acids and three N-linked and one O-linked glycans that is expressed in Chinese hamster ovary cells transfected with DNA encoding HuEPO. HuEPO is the main factor responsible for the proliferation of erythrocytes in the human body. Approximately 90% of HuEPO production takes place in the kidneys whenever a tissue oxygen sensor detects oxygen depletion *(1, 2)*. For patients with kidney failure, the loss of the main HuEPO production site leads to severe anemia, which was treated by blood transfusion until 1988 when rHuEPO became available. Subsequently, rHuEPO has been approved for the treatment of anemia attributable to renal failure, cancer, and other types of anemia. Doping with erythropoietic proteins such as rHuEPO and darbepoetin alfa is perhaps the most complex and serious issue facing sport authorities today. A prodigious amount of anecdotal data indicates that such drugs are widely used in some sports *(3)*.

In endurance sports, the main performance-limiting factor is the oxygen-carrying capacity of the blood. rHuEPO enhances athletic performance by increasing the number of erythrocytes *(4)*. The International Olympic Committee added rHuEPO to its "List of Prohibited Substances" in 1990, although at that time no method existed to detect it in body fluids. A method for detecting rHuEPO in urine by electrophoresis was first described in 1995 *(5)*, and in 2000, Lasne and de Ceaurriz *(6)* introduced an isoelectric focusing (IEF) method coupled with a technique that reduced the nonspecific binding that accompanies immunoblotting. The technique, called "dou-

[1] UCLA Olympic Analytical Laboratory, [2] Department of Family Medicine, Division of Sports Medicine, and [3] Department of Molecular and Medical Pharmacology, University of California at Los Angeles, Los Angeles, CA 90025.

*Address correspondence to this author at: UCLA Olympic Analytical Laboratory, 2122 Granville Ave., Los Angeles, CA 90025. Fax 310-205-9177; e-mail Dcatlin@ucla.edu.

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[4] Nonstandard abbreviations: rHuEPO, recombinant human erythropoietin; IEF, isoelectric focusing; sTfr, soluble serum transferrin receptor; TBR, two-band ratio; QCN, negative quality control; and QCP, positive quality control.

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ble-blotting", led to a practical method that is used to detect rHuEPO and darbepoetin alfa (a long-acting analog of rHuEPO) in the urine of athletes *(7, 8)*.

Very little is known about the time course of urinary rHuEPO after its administration. An increase in immunoreactive EPO has been reported during rHuEPO administration but not in samples collected 4 days after the last dose *(9)*, and Wide et al. *(5)* reported detecting rHuEPO in urine by electrophoresis up to 48 h after the last dose. In this study, our objective was to determine the time course of detectable rHuEPO in urine, using the improved IEF method *(6)*, after nine doses of rHuEPO. We also describe numerical and visual criteria for determining whether rHuEPO is present.

Materials and Methods

DRUGS, REAGENTS, AND CHEMICALS

rHuEPO (Epogen™) was obtained from Amgen Inc. The sources of all other drugs, reagents, and chemicals have been described elsewhere *(7)*.

STUDY PARTICIPANTS

We studied 13 men (9 Caucasians, 3 African Americans, and 1 Asian) and 12 women (11 Caucasians, 1 African American) between 23 and 40 years of age. The study participants were screened by medical history, physical examination, and blood and urine tests. Hematology screening tests included hematocrit, hemoglobin, macrocytes, and percentage reticulocytes. Serum screening tests were creatinine, ferritin, EPO, and soluble transferrin receptor (sTfr). The inclusion criteria included hematocrit of 36–45%, serum ferritin >30 μg/L, and regular physical exercise. Individuals were excluded if the history revealed major organ system disease, substance abuse, participation in any sport drug-testing program, or medications that affect the hematologic, hepatic, or renal systems. Females were screened for pregnancy and African Americans for sickle cell trait. The study was approved by the Institutional Review Board of the University of California at Los Angeles, and all study participants gave written informed consent.

DRUG-FREE CONTROL INDIVIDUALS

We collected one urine from each of 59 men and 37 women between 18 and 44 years of age. The ethnicity of the individuals was 10 African Americans, 7 Asian, 48 Caucasians, 18 Hispanics, and 13 undesignated. Forty-nine individuals lived in Los Angeles, CA, and 47 lived in Salt Lake City, UT. Medical history examination determined that the individuals had no active disease and were not taking any medications affecting the hematologic, hepatic, or renal system. The minimum hematocrits for males and females were 40% and 35%, respectively. The study was approved by the Institutional Review Board of the University of California at Los Angeles, and all study participants gave written informed consent.

PROTOCOL

After 25 individuals qualified for the study, they were paired according to ethnicity, gender, and similar hematologic weighted erythropoietic index *(10)*. One individual in each pair was randomly assigned to the epoetin alfa treatment group and the other to the placebo group. Five individuals who could not be paired were assigned to the epoetin alfa treatment group. The participants were blinded with regard to group. The 15 individuals in the epoetin alfa group (50 IU/kg) and the 10 in the placebo group (1 mL of 9 g/L NaCl) received epoetin alfa or saline subcutaneously between 0800 and 1100 on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. All participants received 325 mg/day of ferrous sulfate (65 mg of elemental iron) by mouth from day −7 to day 25.

Blood and untimed urine samples were collected during the morning of study days −3, 0, 2, 4, 7, 9, 11, 14, 16, 18, 20, 21, 22, and 25. The last 4 study days corresponded to days 2, 3, 4, and 7 postadministration (washout). Blood and urines were collected immediately before administration of saline or epoetin alfa. Blood pressure, pulse, and queries regarding potential side effects were monitored on each visit. If the hematocrit exceeded 48%, the dose for that day was withheld; if the hematocrit exceeded 48% on two or more consecutive visits, the individual was excluded from the study. For all individuals, the red cell indices were measured within 8 h of collection. Serum and urine were stored at −70 °C until analysis. The methods for determining the erythropoietic index have been described *(10)*. In brief, hematocrit, percentage macrocytes, and percentage reticulocytes were determined with an ADVIA120 Hematology Analyzer (Bayer Diagnostics). The serum EPO concentrations were determined with an automated immunometric chemiluminescent assay (Immulite EPO; Diagnostic Products Corporation). The sTfr concentrations were determined by an automated immunonephelometric assay (Dade Behring GmbH). The performance characteristics of the serum EPO and sTfr assays have been described *(11, 12)*.

IEF

IEF was performed as described previously *(7)*. In brief, 20 mL of urine was subjected to ultrafiltration, and the retentate containing EPO was focused in an IEF gel (pH 2–6). The isoforms were double-blotted *(13)* and visualized by chemiluminescence. The emitted light was captured with a FluorChem 8000 CCD camera (Alpha Innotech Corp.).

BAND IDENTIFICATION

A representative example of an IEF gel with two lanes containing markers (lanes S) and four lanes of unknowns (lanes 1–4) is shown in Fig. 1. For the lanes representing samples whose rHuEPO content was unknown, band zero (0) is identified as the band with the same isoelectric point (pI) as the most acidic band in the rHuEPO marker. Starting from band 0, the bands toward the cathode are

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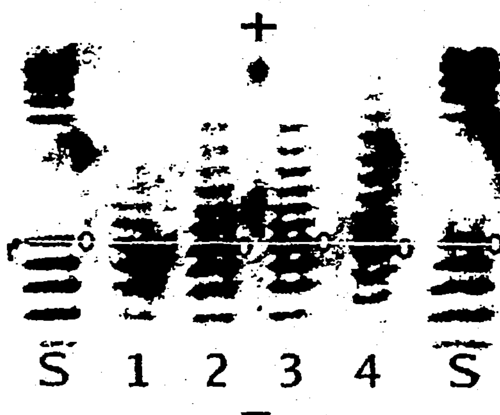

Fig. 1. Example of an IEF image illustrating the selection of the zero band (*0*) for six data lanes.

The two lanes containing markers (*lanes S*) were spotted with 2 fmoles each of rHuEPO and darbepoetin. *Lanes 1–4* are samples obtained from an epoetin alfa-treated individual. The *0* is placed immediately to the *right* of the band corresponding to the most acidic band of rHuEPO in *lanes S*, which in this image is the fifth band from the cathode. The most basic band is faint.

considered "basic" and the bands toward the anode are considered "acidic". Ideally, all bands with the same pI would line up perfectly horizontally across all lanes. In reality, the pH gradient created by ampholytes is subject to disturbances by sample constituents that lead to deflections of the lines of equal pH (iso-pH) and produce bands that have been characterized as "frowns" or "smiles". Smiles are bands with the left and right ends (tips) curved upward, such as band 0 in lane 4 of Fig. 1. Frowns are bands with the tips curved downward (not shown in Fig. 1). As seen in Fig. 1, the horizontal line connecting the 0 bands in the two lanes containing markers (lanes S) also passes through the outer tips of the band labeled 0 in lane 4, leading to the assignment of this band as band 0.

VISUAL DATA ANALYSIS

Three main criteria for identifying rHuEPO were developed. The first criterion was that bands that focus in the basic area of the lane, as determined by the location of the rHuEPO marker, must be darker than other bands in the same lane. The second criterion was that these bands must have the same pI values as the bands in the nearest lane containing a rHuEPO marker. The third criterion was that band 0 and the adjacent two bands in the direction of the cathode must be present. If all three of these visual criteria were met, the sample was considered to contain rHuEPO. If band 0 and the two adjacent basic bands were incompletely resolved or associated with any light or dark spots or imperfections, the sample was reanalyzed. If any of these criteria failed, the sample was categorized "rHuEPO not detectable". Four experienced readers followed the above three criteria to evaluate 48 images of lanes obtained from 24 individuals on days 3 and 7 postadministration. The images were randomized, and the readers were blinded. The instructions were to assign each image to the epoetin alfa or the placebo group.

NUMERICAL DATA ANALYSIS

Lane densitometry was carried out with FluorChem AlphaEase (Ver. 2.01) software (Alpha Innotech Corp), using the 1D-MultiAnalysis, Auto Grid option. The lane template was positioned such that it included two lanes containing markers with a maximum of four unknown lanes in between. For baseline adjustment, the default "Auto Base" method was used, in which the left boundary of the respective lane was broken into 16 regions. The mean background of each of those 16 regions was then used to construct the baseline. The resulting peaks were auto-integrated, using the default settings. Peaks that were not integrated by the software were manually integrated. We defined a peak as one in which the baseline-subtracted height was >30% of its total peak height, i.e., the corresponding band was at least 30% darker than its immediate surroundings. This process is illustrated in Fig. 2, which shows the densitogram of lane 3 of Fig. 1. For each lane in the gel, we calculated a ratio of peak areas. We referred to this ratio as the two-band ratio (TBR) where:

$$TBR = \frac{\Sigma PA_{2\text{-BASIC}}}{\Sigma PA_{2\text{-ACIDIC}}}$$

$\Sigma PA_{2\text{-BASIC}}$ is the combined peak area of the two bands immediately adjacent to band 0 on the basic side, and $\Sigma PA_{2\text{-ACIDIC}}$ is the combined peak area of the two bands adjacent to band 0 on the acidic side. To avoid division by

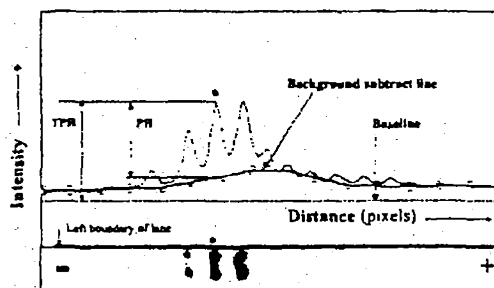

Fig. 2. Densitogram of lane 3 of Fig. 1 showing peaks produced when the CCD camera software integrates the pixel data shown in the electropherogram in Fig. 1.

The x axis represents the distance in pixels from the cathode edge of the integration grid. The y axis represents intensity. The *dashed horizontal line* is the baseline. ⊏—⊐ is the background subtract line determined by the software. *Lane 3*, which has been rotated 90 degrees clockwise from its orientation in Fig. 1, is shown horizontally at the *bottom* of the figure. The left boundary of the lane, which is used to determine the background, is shown schematically as a *solid line* at the *upper edge* of the rotated lane. The + and − represent the anode and cathode. The *starred* (*) peak corresponds to the *starred* band. Total peak height (*TPH*) and background-subtracted peak height (*PH*) are indicated by *arrows*. A peak is accepted if PH is >30% of TPH.

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0, if the latter two bands failed the 30% test, the denominator in the TBR was assigned the value of 1.

STATISTICAL ANALYSIS

All computations were performed on a PC with a Pentium™ chip utilizing the statistical software suite "R" *(14)*. We used the "survival" package with procedure "survreg" to calculate the geometric mean, *x*-axis scale, and various percentiles. Zero values were considered to be left-censored and the distribution to be log-normal. The Wilcoxon rank-sum test was used to determine differences between the epoetin alfa- and placebo-treated groups. *P* values were two-sided, and values <0.05 were considered statistically significant.

Results

CLINICAL EFFECT OF rHuEPO

The baseline characteristics of the placebo and epoetin alfa individuals are shown in Table 1. All individuals were between the ages of 23 and 40 and were well matched for age, weight, height, exercise, and erythropoietic index. The participants completed 347 of the possible 350 visits. Of 135 possible rHuEPO injections, 3 doses were withheld from one individual who was then excluded from the IEF analyses. Two other individuals missed one dose of epoetin alfa but remained in the study.

The efficacy of EPO was established by calculating an erythropoietic index (on-score), which is a weighted index of rHuEPO activity *(10)*. The five variables in the index are hematocrit, reticulocyte hematocrit, percentage macrocytes, serum concentration of EPO, and sTfr. The on-score was calculated using blood samples obtained 3 and 0 days before first drug administration (baseline); samples obtained on days 2, 4, 7, 9, 11, 14, 16, and 18 of treatment; and on days 2, 3, 4, and 7 after the last dose of epoetin alfa or placebo. The mean (SD) on-scores for all females and males in the placebo group on all days were 2.02 (0.045) and 2.27 (0.063), respectively, and there was no change over the 28 days of the study. For the females treated with epoetin alfa, the mean on-score steadily increased to a maximum of 2.77 on day 20 (day 2 postadministration) and then steadily decreased. For the male epoetin alfa group, the mean on-score steadily increased to a maximum of 2.91 on day 18 (last day of epoetin alfa administration) and then steadily decreased. For the male epoetin alfa group, the mean on-score was significantly higher than that of the placebo group for all days after day 2. For the female epoetin alfa group, the mean on-score was significantly higher than that of the placebo group on days 2 and 7–25.

Table 1. Demographic characteristics and baseline data of the individuals who received placebo and epoetin alfa.

	Placebo group		Epoetin alfa group	
	Mean	Range	Mean	Range
Age, years	31.6	23–40	30	23–40
Weight, kg	71.1	55.9–95.9	68.7	53.6–86.4
Height, cm	174	163–190	174	160–190
Exercise, h/week	11.7	5.0–22.5	10.2	5.0–25.0
Creatinine, mg/L	09	08–12	10	08–12
Hematocrit, %	41.8	35.8–44.9	41.2	35.8–44.8
On-score	2.1	1.9–2.3	2.1	1.81–2.38
Male, n	5		8	
Female, n	5		7	
Caucasian, n	9		11	
African-American, n	1		3	
Asian, n	0		1	

REPRESENTATIVE IMAGE OF MARKER AND URINARY ISOFORMS

Shown in Fig. 3 are representative examples of an IEF image obtained from the analysis of markers (lanes S), a negative quality-control urine (lane QCN), a clinical positive quality-control urine (lane QCP), and urines obtained from two epoetin alfa- and one placebo-treated individuals. The Lanes S (markers) contain 2 fmoles each of rHuEPO (the five bands closest to the cathode) and darbepoetin (the four bands closest to the anode).

Also shown in Fig. 3 are the isoform patterns of samples obtained on days 2, 3, and 4 postadministration from individual A in the placebo group (Fig. 3, section A). The placebo-group patterns were very similar to the patterns of the QCN. They were also similar to the patterns observed in the other 9 placebo-treated individuals and the day −3 and 0 samples from the 14 remaining individuals in the epoetin alfa group (not shown). Four samples obtained on days 2, 3, 4, and 7 postadministration from individual B in the epoetin alfa-treated group had patterns very similar to that of the QCP for all days (Fig. 3, section B). Four samples from individual C in the epoetin alfa-treated group, obtained on the same days, had a pattern that changed over time (Fig. 3, section C): close to QCP on day 2, close to QCN on day 7, and intermediate on days 3 and 4. Results for individual C were included in Fig. 3 to show such changes in patterns over time.

VISUAL DATA ANALYSIS

The four experienced readers properly identified the day 3 postadministration samples as being from the epoetin alfa group (n = 14) or the placebo group (n = 10). For the day 7 postadministration samples, the readers properly assigned the 10 placebo samples to the placebo group; however, for the 14 epoetin alfa samples, some were classified as from the placebo group (Fig. 4).

NUMERICAL DATA ANALYSIS

For 19 of the 96 controls, the TBR could not be calculated because of absent peaks (n = 15) and interfering spots (n = 4). Of the remaining 77 samples, 12 (16%) showed a TBR of 0. The 99th percentile was calculated to be 1.19. For the epoetin alfa group, the TBR values ranged from 0.42 to

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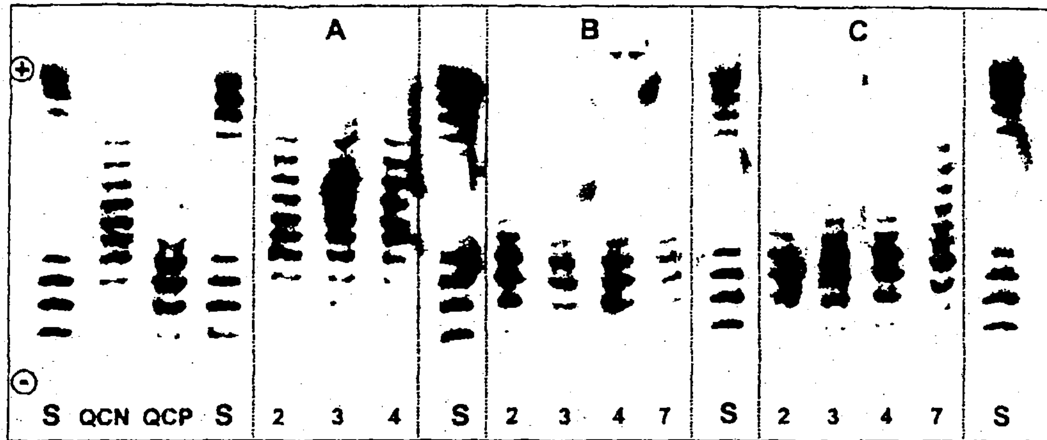

Fig. 3. Representative image of urinary EPO isoforms obtained by IEF analysis of urines from individuals treated with epoetin alfa and placebo.
The five lanes containing markers (*lanes S*) were spotted with 2 fmoles each of rHuEPO and darbepoetin. Lanes *QCP* and *QCN* represent urines from individuals known to be receiving rHuEPO and not to be receiving rHuEPO, respectively. The *lanes* in *section A* were obtained from a placebo-treated individual on postadministration days 2, 3, and 4. The *lanes* in *sections B* and *C* were obtained from epoetin alfa-treated individuals on days 2, 3, 4, and 7.

91 740 on the four postadministration days. None of the samples from the placebo group exceeded a TBR of 1.19 (range, 0–0.77). Of 95 samples analyzed from the epoetin alfa- and placebo-treated individuals on the 4 post-epoetin alfa days, only 3 samples (3%) contained too little EPO for evaluation. Those three samples came from three epoetin alfa-treated individuals on days 4 (n = 1) and 7 (n = 2) postadministration.

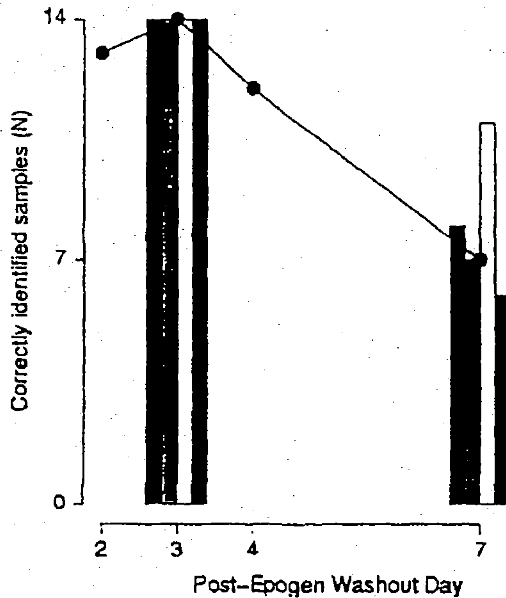

Fig. 4. Number of individuals found to have rHuEPO in their urine 2, 3, 4, and 7 days after the last dose of epoetin alfa.
The *solid lines* connect data points (●) corresponding to the TBR, and the *columns* represent the data determined by visual inspection of the IEF image. The data for the four visual data readers are indicated by *column shading*.

COMPARISON OF THE TBR AND VISUAL METHODS OF DATA ANALYSIS

Shown in Fig. 4 are the differences between the TBR and the visual method of data analysis; Fig. 4 also provides data on the time course of detection of rHuEPO in urine. On post-epoetin alfa days 2, 3, 4, and 7, the TBR properly identified 13, 14 (all), 12, and 7 of the 14 samples, respectively. The visual readers properly identified all the samples on post-epoetin alfa day 3 and between 6 and 11 of the 14 samples on day 7. The percentages of positive results for the two methods of data analysis were identical for the post-epoetin alfa day 3 data and very similar on day 7.

Discussion

The pattern of urinary isoforms of rHuEPO differs from that of endogenous EPO. The former are clustered into four or five bands in the most basic portion of the gel, whereas the latter, which include as many as 14 bands, overlap with and are parallel to the rHuEPO bands in the basic region but are also found in the more acidic region of the gel. Furthermore, after several days of epoetin alfa administration, the endogenous bands become less dense and eventually disappear. These results confirm the findings of Lasne and de Ceaurriz *(6)* and further establish that the method is suitable for detecting rHuEPO in urine.

Our results pertain to epoetin alfa, the drug adminis-

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tered; however, the results would be very similar if epoetin beta were administered. Epoetin beta has been shown to migrate like epoetin alfa, and it has one more band in the basic region *(6)*. It is not possible to comment on the applicability of the method to epoetin omega *(15)* because its pattern of urinary isoforms has not been published.

When rHuEPO is administered to healthy or diseased individuals, it increases the hematocrit, reticulocytes, macrocytes, serum EPO concentration, and sTfr concentration *(16)*. In this study, the increase in the weighted erythropoietic index had reached statistical significance vs the placebo group by day 2, and it continued to increase until it plateaued about day 12. The weighted erythropoietic index did not begin to decrease until the second day after rHuEPO was discontinued and thereafter decreased rapidly but was still greater than the index for the placebo group on day 7 postadministration. This confirms that an indirect index of erythropoietic activity, such as the on-score *(10)*, is a valid indicator of rHuEPO activity.

The presence of rHuEPO in urine followed a similar course. Although endogenous HuEPO contains isoforms that focus in the same area as rHuEPO, there is a significant difference between epoetin alfa and placebo groups in the urinary EPO isoform patterns with respect to the density of the bands within one lane *(6)*. The main difference is where the darkest bands are located. The time course of detection of urinary rHuEPO (Fig. 4) revealed that nearly all of the samples collected on days 2, 3, and 4 after epoetin alfa was discontinued contained rHuEPO. Thus, rHuEPO was highly detectable in urine for up to 4 days after we discontinued epoetin alfa that had been given nine times in 18 days. Between days 3 and 7 after the discontinuation of epoetin alfa, the number of cases detected decreased to ~50%. Therefore, as with many performance-enhancing drugs, the most effective use of this test is in out-of-competition testing. In the urines of individuals B and C, there was hardly any detectable endogenous EPO. The reason for that could be down-regulation of endogenous EPO production *(17, 18)*. It could also be explained by the presence of overwhelming amounts of urinary rHuEPO, which led to the need to dilute the retentate, which in turn would make endogenous EPO harder to detect.

In 1995, Wide et al. *(5)* first reported detecting rHuEPO in urine by electrophoresis. Their assay was sufficient to detect rHuEPO in the urine of 11 of 11 individuals in samples collected up to 24 h after rHuEPO was administered (20 IU/kg three times per week) for 7–9 weeks. The detection rate decreased to 75% at 48 h. Urinary EPO has also been detected by RIA during rHuEPO administration (200 IU/kg on 5 of 10 days) but not 4 days after the last dose *(8)*. In the current study, the last collection day was day 7 postadministration, and approximately one-half of the participants still had detectable urinary rHuEPO.

The visual and numerical methods for evaluating IEF data produced very similar results (Fig. 4). Four different data readers, using the three visual criteria, correctly identified the 14 individuals in the epoetin alfa group and the 10 individuals in the placebo group on samples collected 3 days after the last dose of epoetin alfa. On day 7 postadministration, when the amount of rHuEPO was expected to be decreasing and the serum concentrations of EPO had decreased *(16)*, the readers still detected rHuEPO in ~50% of the samples, although with a lesser degree of concordance.

Densitometry converts band patterns into numerical values that can be readily combined into various scores or ratios. Because the isoforms of HuEPO and rHuEPO focus in overlapping areas, whatever scores or ratios are used must account for the usual variation of HuEPO isoform patterns. We selected the TBR as our preferred method of numerical analysis after evaluating various other ratios that could be used to characterize urinary rHuEPO. Compared with numerical methods that require analyzing all of the bands, the advantages of the TBR method include simplicity (only four bands need to be analyzed), speed (the analysis and calculation times are considerably reduced), fewer lanes declared invalid because of interfering spots, and easier accommodation of lanes that are not in perfect vertical alignment. In addition, there is no ambiguity regarding assignment of band 0.

In our procedure, the isoform pattern is visually inspected and the three criteria described here are applied. Samples that do not meet these criteria are declared "no detectable rHuEPO". If the criteria are met but some bands overlap or have interfering spots, the samples are reanalyzed. The TBR is then determined by lane densitometry. If both the visual and numerical criteria are met, it is highly likely that the sample contains rHuEPO. From the perspective of statistical limitations, at this time our numerical data are sufficient to determine the 99th percentile. The 99.9th percentile or higher margins of safety require a larger number of samples. Accordingly, we believe it is advisable to accumulate more data and to participate in interlaboratory studies designed to enhance the criteria for stating that a sample contains rHuEPO.

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Comparison of the Isoelectric Focusing Patterns of Darbepoetin Alfa, Recombinant Human Erythropoietin, and Endogenous Erythropoietin from Human Urine, *Don H. Catlin,*[1,3*] *Andreas Breidbach,*[1] *Steve Elliott,*[2] *and John Glaspy*[3] ([1] UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, and [3] Department of Medicine, University of California, Los Angeles, CA 90025; [2] Amgen Inc., Thousand Oaks, CA 91320-1799; * address correspondence to this author at: UCLA Olympic Analytical Laboratory, 2122 Granville Ave., Los Angeles, CA 90025; fax 310-206-9077, e-mail dcatlin@ucla.edu)

Novel erythropoiesis-stimulating protein (Aranesp™; darbepoetin alfa) is a glycoprotein hormone with a longer serum half-life than recombinant human erythropoietin (rHuEPO) *(1)*. The polypeptide backbone of the human EPO molecule has an invariant amino acid sequence; however, the carbohydrate side chains exhibit microheterogeneity in sugar content and structure *(2–4)*. A negatively charged sialic acid molecule typically caps the end of each arm of a carbohydrate chain. As a consequence, the variable nature of the sialic acid content gives rise to EPO isoforms with differences in charge *(3)*. After purifying isoforms of rHuEPO, Egrie and coworkers *(5, 6)* discovered a direct correlation between the number of sialic acid groups on the carbohydrate part of rHuEPO and both its serum half-life and biological activity, as well as an inverse relationship with receptor binding. These data showed that pharmacokinetic factors have a greater influence on biological activity than receptor binding affinity. These principles explain the increased half-life and increased in vivo activity of darbepoetin alfa, which contains 5 N-linked carbohydrate chains and up to 22 sialic acids *(5, 7)*. In contrast, rHuEPO has 3 N-linked carbohydrate chains and a maximum of 14 sialic acids *(5, 7)*.

Similar clinical responses can be achieved by administering darbepoetin alfa once a week or rHuEPO three times a week *(8, 9)*. The efficacy of darbepoetin alfa in the treatment of anemia associated with chronic renal failure has been shown *(10)*, and in 2001 it was approved by the US Food and Drug Administration for that indication. Darbepoetin alfa is under investigation for the treatment of anemia in cancer patients *(11)* and other applications. Although darbepoetin alfa was approved only recently, we detected darbepoetin alfa in the urine of three athletes competing in the 2002 Winter Olympic Games in Salt Lake City. To date, it has not been reported in human urine.

The isoelectric focusing (IEF) patterns of standard rHuEPO, endogenous human EPO in urine extracts, and administered rHuEPO in urine extracts have been reported *(12)*. This report describes the IEF pattern observed after applying the same method to standard darbepoetin alfa and post-administration urine extracts.

The pooled urine of two healthy, drug-free males was used as the endogenous HuEPO control urine (QC1). The rHuEPO positive control urine (QCP) was pooled urine from healthy individuals (eight males and seven females) who received rHuEPO on nine visits over 19 days (50 IU/kg at each visit). Some, but not all, urines were included in the pool. A urine collected from a female cancer patient 1 week after a single dose (0.675 μg/kg) of darbepoetin alfa (Aranesp; Amgen Inc., Thousand Oaks, CA) was used as the darbepoetin alfa control urine. The participants gave written informed consent under applications approved by the UCLA Office of Human Subject Protection.

Aranesp (60 mg/L) containing human serum albumin was obtained from a pharmacy. EPO Biological Reference Preparation (BRP) was obtained from the European Directorate for the Quality of Medicines (Strasbourg, France). Tris base, phosphate-buffered saline tablets, glycine, 100 mL/L Tween 80R (low peroxide), dithiothreitol, sucrose, and bovine serum albumin (RIA grade) were purchased from Sigma. Protease inhibitor (Complete) was purchased from Roche Diagnostics. Urea, Ready-Mix IEF acrylamide/bisacrylamide (29:1 by weight), ammonium persulfate, and *N,N,N,N*-tetramethylethylenediamine were purchased from Amersham Biosciences, and the ampholytes Servalyt 2-4, 4-6, and 6-8 were purchased from Serva. Nonfat dry milk was purchased in a supermarket. The primary antibody (AE7A5; monoclonal mouse anti-hEPO) was obtained from R&D Diagnostics, and the secondary antibody conjugate [biotin–goat anti-mouse IgG (H+L)] and horseradish peroxidase–streptavidin conjugate (both Zymax grade) were obtained from Zymed Laboratories. The chemiluminescence substrate (ChemiGlow) was obtained from Alpha Innotech Corp. Phosphoric acid was obtained from Aldrich Chemicals, glacial acetic acid (HPLC grade) was from Mallinckrodt Chemical, and black ink (Tusche A) was from Pelikan.

Unless specified, we used electrophoresis or higher grade chemicals.

The method was originally described by Lasne *(13)*. All modifications are detailed below. A minimum of 20 mL of urine was adjusted to near neutral pH with 3.75 mol/L Tris (pH 7.4) to inhibit any acidic protease activity. The activities of other proteases were inhibited by adding Complete. Any particulate matter was removed from the urine by centrifugation and microfiltration (0.22 μm) of the supernatant. The filtrate was reduced to the smallest possible retentate volume with a two-step ultrafiltration [Millipore Centricon Plus-20 + Centricon YM-30 (molecular weight cutoff, 30 000)]. The volume reduction included one washing step with 50 mmol/L Tris (pH 7.4) and Complete. The final retentate (20 μL) was applied to an IEF gel after adjustment of the apparent EPO concentration to a maximum of 500 IU/L.

A polyacrylamide gel (250 × 120 × 1 mm; 5% T, 3% C; 50 g/L sucrose, 50 mL/L Servalyt 2-4, 50 mL/L Servalyt 4-6, 7 mol/L urea) was prefocused for 30 min at 250 V and 8 °C, with 50 mL/L Servalyt 6-8 as the catholyte and 0.5 mol/L H_3PO_4 as the anolyte. We then applied 20 μL of either a 0.1 nmol/L standard (EPO BRP or Aranesp) or the urine extracts (heat inactivated for 3 min at 80 °C) containing 10 mL/L Tween 80R approximately 5 mm from the cathode. The gel was focused for 4000 Vh with maximum settings of 2000 V, 50 mA, and 30 W.

The focused proteins were detected by "double-blotting" *(13)*. In this procedure, in which the primary antibody (monoclonal mouse anti-hEPO) is electroblotted (1 mA/cm^2 for 10 min) to a second membrane, nonspecific binding of the secondary antibody [biotin-goat anti-mouse IgG (H+L)] is markedly decreased. After incubation with streptavidin–horseradish peroxidase and ChemiGlow substrate, the emitted light was captured with a chemiluminescence imaging system (FluorChem 8000; Alpha Innotech Corp.).

An isoform of EPO is a subset of the EPO molecules that has a defined charge. The isoforms appear in the electropherogram as bands. An isoform pattern consists of bands, specifically their number, positions, and densities relative to each other. The number of isoforms and their

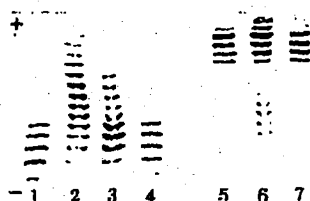

Fig. 1. Electropherogram of rHuEPO and darbepoetin alfa standards and extracts of urine obtained from healthy controls and individuals treated with rHuEPO and darbepoetin alfa.

The anode and cathode sides of the electropherogram are indicated as + and −, respectively. *Lanes 1* and *4*, rHuEPO standard; *lanes 5* and *7*, darbepoetin standard; *lane 2*, extract from a healthy individual showing the normal pattern of endogenous hEPO; *lanes 3* and *6*, urine extracts obtained after the administration of rHuEPO and darbepoetin alfa, respectively.

positions result directly from the structural characteristics of the molecules.

The number of charged molecules, such as the sialic acid content of the carbohydrate, influences the isoelectric point (pI), which in turn determines the final position of the isoform on the gel. Within one lane, the denser the isoform, the more of that particular isoform is present in that lane.

Fig. 1 is an electropherogram showing the patterns of isoforms from rHuEPO and darbepoetin alfa standards, endogenous urinary EPO, and administered rHuEPO and darbepoetin alfa. The isoform pattern of a urine extract from QC1 (Fig. 1, lane 2) contained at least 10 isoforms. The isoforms closest to the anode and cathode are less dense than the isoforms in the middle.

As predicted from the chemical differences between rHuEPO and darbepoetin alfa standards, the migration patterns and pIs of rHuEPO and darbepoetin alfa differed greatly. Darbepoetin alfa appeared in the anodic region, and there was no overlap with rHuEPO, which appeared in the cathodic region.

The isoform pattern of pharmaceutical darbepoetin alfa is shown in lanes 5 and 7 (Fig. 1). It contains four dominant isoforms clustered in the acidic area of the electropherogram. Isoform density increases from the least to the most acidic band. The isoform pattern of an extract of a urine from a cancer patient who received darbepoetin alfa (Fig. 1, lane 6) matched that of pharmaceutical darbepoetin alfa in terms of the number of isoforms, their positions, and their relative intensities. The match establishes the identity of the compound in the urine extract (Fig. 1, lane 6) as darbepoetin alfa.

Although there are faint isoforms of endogenous EPO in the anodic region (Fig. 1, lane 2), the density in this region is minimal, and the overall isoform pattern is distinctly different from that of the darbepoetin alfa lanes. In contrast to the isoforms of the darbepoetin alfa standard (Fig. 1, lanes 5 and 7), the isoforms of the EPO BRP standard (lanes 1 and 4) are in the less acidic area of the electropherogram. The pattern of isoforms in urine obtained after rHuEPO was administered to individuals is shown in lane 3. This pattern is characterized by very dense isoforms in the least acidic area and lighter isoforms moving toward the anode.

In our experience with electrophoresis performed on urines, obtained from >300 healthy control individuals, lane 2 is a typical normal pattern, which was first published by Lasne and de Ceaurriz *(12)*. This work demonstrates that both rHuEPO and darbepoetin alfa appear in the urine. Differences in the isoform patterns of these pharmaceuticals compared with endogenous (urinary) EPO are readily apparent. The fact that a strong darbepoetin alfa signal is observed in a urine sample from a patient 7 days after administration of the drug is consistent with its mean terminal half-life of 25.3 h *(1)*.

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Neopterin Concentrations in Cord Blood: A Single-Cohort Study of Paired Samples from 541 Pregnant Women and Their Newborns, *Harald Schennach,[1] Christian Murr,[4] Clara Larcher,[5,6] Werner Streif,[2] Erika Pastner,[3] Daniela Zaknun,[7] Diether Schönitzer,[1] and Dietmar Fuchs[4,6*]* ([1] Central Institute for Blood Transfusion, and Departments of [2] Pediatrics and [3] Gynecology, University Hospital Innsbruck, A-6020 Innsbruck, Austria; Institutes of [4] Medical Chemistry and Biochemistry and [5] Hygiene and Social Medicine, Leopold-Franzens University, and [6] Ludwig Boltzmann Institute for AIDS Research, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria; [7] Department of Pediatrics, University of Vienna, A-1090 Vienna, Austria; [*] address correspondence to this author at: Institute of Medical Chemistry and Biochemistry, Leopold-Franzens University, and Ludwig Boltzmann Institute for AIDS Research, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria)

Neopterin, a product of interferon-γ-activated monocyte-derived macrophages, is a sensitive indicator of cell-mediated immune activation *(1)*. In humans, increased concentrations of neopterin in serum and urine have been found in various malignant disorders and autoimmune diseases as well as during allograft rejection episodes and viral infections, including HIV type 1 *(2–8)*. Serum neopterin concentrations have also been investigated during pregnancy and in the neonatal period *(9–11)*.

In this study, serum neopterin was measured in women with uncomplicated pregnancies, and concentrations were compared with cord-blood concentrations after delivery. A total of 541 women with a median age of 29.0 years (range, 15.5–44.3 years) who delivered at the University Hospital Innsbruck between October 1997 and July 1999 and who had all examinations during pregnancy performed at the same institution were included in the study. All of them took part in the Austrian healthcare program called "Mutter-Kind-Pass", which is recommended to every pregnant woman and is supported by the public health system. This program includes at least five gynecologic examinations and one internal medical investigation during pregnancy. In addition, all pregnant women are tested for antibodies against rubella virus, *Treponema pallidum*, and *Toxoplasma gondii* and are screened for hepatitis B surface antigen. None of them had medical or obstetric complications. All pregnancies were uncomplicated singleton gestations that produced (with one exception) healthy term infants (290 males and 251 females), whose growth was appropriate for gestational age. In keeping with customary healthcare practice in Austria, the development of all the children was checked by medical investigations at least five times beginning with the neonatal period up to the age of 14 months. In addition to this routine program, EDTA-blood samples collected from all newborns by heel lancing in the first week after birth were tested for cytomegalovirus (CMV) by the qualitative Amplicor CMV test (Roche Molecular Systems). This PCR assay amplifies a 365-bp fragment of the CMV polymerase gene and has a limit of detection of ~1000 copies/mL *(12)*.

Blood samples were drawn by venipuncture of the mother in the 28th week of gestation. Immediately after delivery, blood samples were drawn by puncture of the umbilical artery of the cord before the placenta was discarded. The blood was allowed to clot at room temperature, and serum was obtained by centrifugation at 3220*g* for 15 min. Neopterin analyses were performed within 1 day after blood collection. Serum neopterin was measured by a commercially available ELISA (ELItest® Neopterin; BRAHMS Diagnostica) with a detection limit of 1 nmol/L neopterin and an interassay CV ranging from 3.9% to 8.2% *(13)*. Upper reference limits (95th percentiles) for neopterin concentrations are age-dependent and range from 8.7 nmol/L (19–75 years) to 13.5 nmol/L (<19 years) and 19.0 nmol/L (>75 years) as described previously *(13)*. The study was approved by the local ethics committee, and consent was obtained from all participating women before all procedures were performed.

Correlation between variables was assessed by the nonparametric Spearman rank correlation method because the distributions of observed values were generally

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