Published in final edited form as:

Vet Anaesth Analg. 2013 July ; 40(4): 440–448. doi:10.1111/vaa.12030.

Use of a soluble epoxide hydrolase inhibitor as adjunctive analgesic in a laminitic horse

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Abstract

A 4-year old, 500 kg Thoroughbred female horse diagnosed with bilateral forelimb laminitis and cellulitis on the left forelimb became severely painful and refractory to non-steroidal anti-inflammatory therapy (flunixin meglumine on days 1, 2, 3 and 4; and phenylbutazone on days 5, 6 and 7) alone or in combination with gabapentin (days 6 and 7). Pain scores assessed independently by three individuals with a visual analog scale (VAS; 0= no pain and 10=worst possible pain) were 8.5 on day 6, and it increased to 9.5 on day 7. Non-invasive blood pressure monitoring revealed severe hypertension. As euthanasia was being considered for humane reasons as well as technical and financial constraints, a decision was made to add an experimental new drug, *trans*-4-{4-[3-(4-Trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (*t*-TUCB), which is an inhibitor of soluble epoxide hydrolase (sEH), to the treatment protocol. Dose and frequency of administration were selected to produce plasma concentrations within the range of 2.5 μ M and 30 nM based on the drug potency against equine sEH. Pain scores decreased sharply and remarkably following *t*-TUCB administration and blood pressure progressively decreased to physiologic normal values. Plasma concentrations of *t*-TUCB, measured daily, were within the expected range, whereas phenylbutazone and gabapentin plasma levels were below the suggested efficacious concentrations. No adverse effects were detected on clinical and laboratory examinations during and after *t*-TUCB administration. The mare did not get any episode of laminitis in the three months following the treatment.

Introduction

Laminitis is an extremely painful condition of the foot in horses. Its pathophysiology remains poorly understood, but involves both vascular and inflammatory events within the hoof leading to disruption of the lamellar dermo-epidermal junction, impaired biomechanical function, pain and substantial suffering (Hood et al. 1993; Hood 1999; Sumano Lopez et al. 1999; Parks & O'Grady 2003; Driessen et al. 2010). Ischemia and inflammation in the early stages of laminitis likely cause neuronal injury that eventually shifts the acute inflammatory pain into a chronic syndrome with a prominent neuropathic component (Moalem & Tracey 2006; Peroni et al. 2006; Belknap et al. 2007; Jones et al. 2007). The precise timing and nature of these events remain elusive. The response to

treatment can be quite unpredictable. Such complexity makes pain management in horses with laminitis one of the biggest challenges in equine practice. Non-steroidal anti-inflammatory drugs (NSAID) are the mainstay analgesics for this condition. However, abridged efficacy against neuropathic pain and risks of dose-dependent gastrointestinal and renal adverse effects are significant limitations of these compounds (Sumano Lopez et al. 1999; Taylor et al. 2002; Driessen et al. 2010). These constraints often leave euthanasia as the only humane alternative to alleviate pain and suffering in affected horses (Driessen et al. 2010). This clearly underscores the need for the development of more efficacious and safer analgesics.

The oxidative metabolism of polyunsaturated fatty acids (PUFAs) such as arachidonic acid (ARA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and linoleic acid (LNA) produces potent inflammatory mediators. Most of the analgesic research and drug development has focused on inhibiting ARA derivatives formed by cyclooxygenases (COX) (Tokuyama & Nakamoto 2011). Cytochrome P450 enzymes mediate another critical yet relatively unexplored pathway of PUFAs metabolism. This pathway transforms PUFAs into various biologically active compounds, including epoxy-fatty acids (EFAs or epoxides), such as epoxy-eicosatrienoic acids (EETs), or hydroxyl derivatives, such as hydroxy-eicosatetraenoic acids (HETEs) (Wagner et al. 2011b). These EFAs have multiple biological activities including the modulation of inflammation and nociceptive signaling (Murakami 2011). The biological activity of these epoxides is restricted as they are metabolized to the corresponding diols by the soluble epoxide hydrolases (sEH) (Wagner et al. 2011a). This has been confirmed with the development and use of sEH inhibitors (sEHIs) (Morisseau & Hammock 2005; Hwang et al. 2007) in conditions involving several body systems and functions (Revermann 2010). The microsomal (mEH) and soluble (sEH) epoxide hydrolases were first thought to play a role in xenobiotic metabolism in mammalian tissues. Even though this is largely true for mEH, sEH has a minor role in xenobiotic metabolism. The major function of sEH is the degradation of endogenous lipid metabolites (Morisseau & Hammock 2008; Decker et al. 2009).

In the horse, sEH has been characterized in the liver and lungs (Lakritz et al. 2000), but its biological roles have yet to be examined in vivo. Several lines of evidence from studies in classic rodent models of inflammatory and neuropathic pain (Inceoglu et al. 2006; Schmelzer et al. 2006; Inceoglu et al. 2007; Inceoglu et al. 2008; Morisseau et al. 2010; Wagner et al. 2011a; Wagner et al. 2011b) suggest that analgesia is likely to be produced via sEH inhibition in horses with pain due to laminitis (Sumano Lopez et al. 1999; Jones et al. 2007; Driessen et al. 2010). In a rat model of inflammatory pain, transdermal administration of two distinct sEHIs effectively attenuated LPS-induced thermal hyperalgesia and mechanical allodynia (Inceoglu et al. 2006). Similarly positive results were obtained in other models of inflammatory and neuropathic pain (Inceoglu et al. 2007; Inceoglu et al. 2008). In fact, these compounds are stronger anti-inflammatory and analgesic drugs in rodent models than coxibs or NSAIDs (Inceoglu et al. 2007; Wagner et al. 2011b). Enhancement of anti-nociception by co-administering low dose of several NSAIDs with sEHIs was also demonstrated in a mouse model of inflammatory pain. The sEHI administered alone decreased COX-2 protein expression and prostaglandin E₂ (PGE₂) production in LPS-treated mice with no effect on COX-1 expression. When NSAIDs and sEHIs were combined, inhibition of COX-2 protein expression was enhanced with reduction in PGE₂ concentrations without disrupting prostacyclin and thromboxane levels (Schmelzer et al. 2006). These findings along with observations that sEHIs reduce pain induced by direct intra-plantar injection of PGE₂, whereas NSAIDs and steroids do not, indicate that these drugs have distinct mechanisms of action that can be useful in multimodal therapies (Inceoglu et al. 2011).

It has been proposed that sEH-mediated anti-hyperalgesia in inflammatory and neuropathic pain occurs via two distinct mechanisms. One mechanism involves direct anti-inflammatory action of epoxides including down-regulation of induced cyclooxygenase (COX)-2 expression, possibly through a nuclear factor-kappa B (NF- κ B)-dependent pathway. Such mechanism mimics the analgesia by non-steroidal anti-inflammatory drugs (NSAIDs) but as transcriptional regulators instead of direct enzyme inhibitors. The second mechanism involves epoxide-mediated up-regulation in steroid/neurosteroid synthesis in the presence of elevated cAMP levels, which then results in analgesia via GABA channels (Inceoglu et al. 2008). Collectively, the multimodal mechanism of action and the favorable interactions with NSAIDs in the ARA cascade suggest that sEH and COX inhibitors combinations may produce significant pain relief while minimizing the risks of NSAID-associated side effects.

In this case report, we describe the use of an experimental sEH inhibitor as analgesic adjunct in a horse with laminitis.

Case presentation

On November 2, 2011, a 4-year-old, 500kg, female Thoroughbred horse was examined by the Veterinary Field Service of the UC Davis Veterinary Medical Teaching Hospital with the presenting complaint of swollen on the left forelimb and reluctant to walk. The mare was reportedly found that morning unable to move, painful in both front feet and a rectal temperature of 38.6 °C. Pertinent previous history included a moderate to severe lesion (44% tear) of the left forelimb superficial digital flexor tendon while on the racetrack in April 2011. The mare was then donated to the UC Davis Center for Equine Health to be used as a research subject, and was subsequently enrolled in a stem cell study. In July 2011, the mare underwent computed tomography angiography followed by intraarterial regional limb perfusion of ^{99m}Tc -HMPAO labeled mesenchymal stem cells of the left forelimb. The cells were delivered via a catheter placed in the median artery at the level of the distal radius and the perfusion was performed without the use of a tourniquet. Swelling of the region of the left carpus and proximal region of the third metacarpal bone was noted in the immediate post-operative period. It resolved without obvious complications following treatment that included leg bandage, stall rest, and oral administration of phenylbutazone (1g twice a day) for three days.

On Nov. 2nd, physical examination findings included tachycardia (60 beats minute^{-1}), tachypnea (40 breaths minute^{-1}), increased digital pulses in both forelimbs, bilateral forelimb swelling in the region of the third metacarpal bones, focal swelling on the medial and lateral aspect of the left radius that appeared painful on palpation. The mare had symmetrical adequate muscling but was standing in a rocked back position and unable to walk without much encouragement. All other physical examination parameters were within normal reference limits. Orthogonal radiographic projections of the left and right front distal extremities revealed medial to lateral hoof imbalance bilaterally, mild dorsal hoof wall thickening bilaterally, with fracture of the dorso-distal aspect of the distal phalanx bilaterally, but no evidence of rotation or sinking. Orthogonal and craniolateral-caudomedial oblique radiographic projections of the left radius revealed radial soft tissue swelling without evidence of osseous involvement. Irregularity of the caudodistal left radius likely represented remodeling secondary to previous trauma unlikely to be clinically significant. The changes in the distal extremities were suggestive of laminitis.

A clinical diagnosis of left forelimb cellulitis and bilateral forelimb laminitis was made. Initial therapy included cold hydrotherapy (once daily), flunixin meglumine (1 mg kg^{-1} , twice daily, IV), penicillin G procaine (PPG; 24,000 U kg^{-1} , twice daily, IM), gentamicin (3.5 mg kg^{-1} , once daily, IV). Soft Ride boots were applied bilaterally. A sweat stack wrap

was placed on the left forelimb, and a standing sweat wrap was placed on the right, both containing furazone, dimethyl sulfoxide (DMSO) and epsom salts. This therapy was continued for the next four days (days 2, 3, 4 and 5), although the dose and route of administration of flunixin meglumine were changed (0.5 mg kg^{-1} , twice daily, PO) on days 4 and 5. Over this period, the cellulitis was improving and the mare appeared more comfortable until day 5, when it became increasingly more painful. Phenylbutazone (4 mg kg^{-1} , IV) was administered for pain relief and the therapy was changed such that flunixin meglumine and PPG were discontinued and phenylbutazone (4 mg kg^{-1} twice daily, PO), trimethoprim sulfamethoxazole (30 mg kg^{-1} twice daily, PO) and pentoxifylline (11 mg kg^{-1} twice daily, PO) were instituted. On day 6, the mare became very painful (pain score 8.5/10 on visual analog scale, VAS; “0” = no pain and “10” = worst possible pain (Vinuela-Fernandez et al. 2011) and was standing but unwilling to walk. Although the cellulitis had improved significantly (almost not noticeable), the mare was displaying signs of laminitis in both forefeet. The hind feet also appeared to be slightly painful. Gabapentin (20 mg kg^{-1} twice daily, PO) was added to the treatment protocol.

The condition further deteriorated on day 7, with the mare spending most of the day laying down in lateral recumbency. It required much encouragement to stand up and, once standing, was unwilling to walk. At this time, systematic assessments of pain with the use of a VAS, as well as monitoring of blood pressure, heart and respiratory rates, and gastrointestinal sounds (Teixeira Neto et al. 2004) were instituted. Blood pressure was measured in triplicates, non-invasively with oscillometric technique (Cardell Model 9401 BP Monitor, Sharn Veterinary Inc., Tampa, FL.), with a tail cuff (width equal to 40% of the circumference of the base of the tail) and the horse in standing position (corrected for heart-tail height difference). Three individuals (two third year residents - equine surgery and anesthesia - and one board certified anesthesiologist) independently assessed the patient throughout the day taking into account changes in expression, demeanor, posture, stance and mobility. Two individuals (residents) were unaware of the identity and mechanism of action of the compound. An overall daily VAS score was then assigned. All assessments were done with the patient in the stall and the results are shown in Figure 1. On day 7, the average VAS score was 9 out of 10 and blood pressure measurements revealed significant hypertension.

Euthanasia was being considered at this stage for humane reasons coupled with technical and financial constraints. A decision was made to add an experimental drug, *trans*-4-{4-[3-(4-Trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (*t*-TUCB), to the treatment protocol. This drug has been shown to be a potent analgesic in classic rodent models of inflammatory and neuropathic pain (Inceoglu et al. 2006; Schmelzer et al. 2006; Inceoglu et al. 2007; Inceoglu et al. 2008; Morisseau et al. 2010; Wagner et al. 2011a; Wagner et al. 2011b), and is currently being investigated as a potential new analgesic in horses under approval by the Institutional Animal Care and Use Committee of the University of California-Davis. Dose (0.1 mg.kg^{-1}) and frequency of administration (once daily) were selected to produce plasma concentrations within the range of approximately $2.5 \text{ }\mu\text{M}$ (peak) and 30 nM (trough). Concentrations in this range are expected to be sufficient to inhibit the equine sEH *in vivo* on the basis of previous studies (Inceoglu et al. 2006; Morisseau et al. 2006; Inceoglu et al. 2008; Tsai et al. 2010; Ulu et al. 2011) and the *in vitro* potency against the equine sEH (unpublished data). The drug was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg.ml^{-1} , filter-sterilized with $0.2 \text{ }\mu\text{m}$ pore size sterilizing-grade membranes, and administered intravenously as a bolus by hand over a period of approximately one minute. To determine the plasma concentrations of *t*-TUCB, blood samples were collected from the opposite jugular vein just prior to *t*-TUCB administration (baseline), at 5, 15 and 30 minutes, and at 1, 2, 4, 8, 12 and 24 hours following each of the first three doses (days 8, 9 and 10), at 6, 12, 18 and 24 hours following each of the next two doses (days 11 and 12), and at 36, 48, 72 and 96 hours following the

last dose (day 12). Plasma concentrations of phenylbutazone and gabapentin were determined in these same blood samples, but corresponded to slightly different time points since they were being administered one hour after (phenylbutazone) or five hours before (gabapentin) *t*-TUCB. The results are shown in Figure 2. In addition, blood was also collected on days 8, 9, 10 and 13 for laboratory analyzes of complete blood cell count (CBC) and serum biochemistry (CHEM) and results are presented on Table 1.

The first dose of *t*-TUCB was administered early in the morning of day 8. The mare spent majority of that day standing in the stall, was interested in surroundings, begun to walk spontaneously and was frequently looking out the front stall door. The average VAS pain score was 5.5. Hypertension was still present. Initial laboratory analyzes of CBC and CHEM revealed no significant changes after the first dose of *t*-TUCB. With these encouraging results, *t*-TUCB continued to be administered for four more days (days 9, 10, 11 and 12). In the following days, the mare continued to improve in expression, demeanor, posture, stance and mobility, which was reflected by lower VAS pain scores (Figure 1A). As treatment progressed, the hypertension improved gradually towards normal physiologic values (Figure 1B).

Daily plasma concentrations of *t*-TUCB were within the expected range, although it did not reach 2.5 μM and it fell below 30 nM on one occasion in the first day. The calculated volume of distribution, elimination half-life and clearance of *t*-TUCB for this horse were 1.22 ml kg⁻¹, 29.8h and 0.04 ml h⁻¹ kg⁻¹, respectively. The highest and lowest measured plasma concentrations of phenylbutazone were 55 μM and 2 μM , and those of gabapentin were 18 μM and 1 μM . The true peaks of gabapentin were likely missed since the first blood sampling occurred five hours after dosing. This was due to the scheduled times for gabapentin administration and because the primary goal in this case was to determine the plasma concentrations of *t*-TUCB.

No adverse effects were observed both in the clinical exams and evaluation of blood work (Table 1). At 30 days follow-up the mare was normotensive and had no evidence of lameness. At 90 days, a few irregularities were apparent on the hoof wall, no episodes of lameness have been noticed.

Discussion

This case report is the first description of the successful use of the sEH inhibitor *t*-TUCB, as analgesic adjunct in a horse with laminitis. The horse was being treated for laminitis for seven days and, after an initial improvement, the condition deteriorated significantly. The severe pain was not responding to NSAIDs and gabapentin therapy. A remarkable reduction in pain scores occurred after pharmacological inhibition of sEH with *t*-TUCB. Notably, after being recumbent most of the previous day, the horse stood after the first dose of *t*-TUCB and was willing to walk, albeit somewhat reluctantly, in the stall and had good appetite. Inhibitors of sEH have been shown to be potent anti-inflammatory and analgesic agents in classic rodent models of both inflammatory and neuropathic pain (Inceoglu et al. 2006; Schmelzer et al. 2006; Inceoglu et al. 2007; Inceoglu et al. 2008; Morisseau et al. 2010; Wagner et al. 2011a; Wagner et al. 2011b). The observations in this horse with naturally occurring laminitis suggest that these compounds work not only in experimental models, but may have utility in the treatment of diseases associated with inflammation and pain. This notion is being tested in ongoing experiments. Preliminary data show that the concentration of several epoxides and respective diols derived from relevant long-chain fatty acids is changed in laminitic compared to healthy horses (unpublished data).

Inhibitors of sEH have been shown to be stronger anti-inflammatory and analgesics than coxibs or NSAIDs in rodent models of inflammatory pain (Inceoglu et al. 2007; Wagner et al. 2011b). Thus, it is not possible to ascertain the sole analgesic contribution of *t*-TUCB in the case reported here even though pain that was refractory to phenylbutazone and gabapentin promptly improved once the sEHI was administered. Interactions of *t*-TUCB with phenylbutazone, gabapentin and/or pentoxifylline likely occurred. It is known that co-administration of NSAIDs and sEHIs result in enhancement of antinociception (Schmelzer et al. 2006). Interestingly, measured phenylbutazone plasma levels were below its 80% maximal inhibitory concentration (IC₈₀; ~23 µM) against COX-2 for most of the time. The IC₈₀ rather than the IC₅₀ value seems more suitable for NSAIDs evaluation, particularly since valid anti-inflammatory effects are achieved when COX-2 activity is 80% inhibited (Beretta et al. 2005). However, an additive or even a synergistic effect between *t*-TUCB and phenylbutazone could be responsible for the analgesic efficacy in this report.

The minimum effective analgesic plasma concentration of gabapentin in horses is unknown (Terry et al. 2010) although it was used successfully in one horse with neuropathic pain (Davis et al. 2007). Measured plasma concentrations in the horse of the present report were well below the concentration that has been associated with analgesia (~18 µM) in human volunteers (Eckhardt et al. 2000). There probably was a positive interaction with the phosphodiesterase inhibitor pentoxifylline since analgesia produced by sEHIs is cyclic AMP (cAMP)-dependent, other phosphodiesterase inhibitors have been shown to increase EET concentrations (Inceoglu et al. 2011), and pentoxifylline itself may have analgesic effects in inflammatory and neuropathic pain states (Vale et al. 2004; Liu et al. 2007). Collectively, the above information corroborate with the conclusion that sEH inhibition with *t*-TUCB played a central role in the pain management of the horse of this report. The favorable interactions between sEHIs and NSAIDs in the arachidonic acid cascade might allow for the use of lower doses of NSAIDs while maintaining efficacy and minimizing the risks of NSAID-associated side effects.

Drugs or techniques that provide complete control of nociception are not desirable in horses with laminitis because pain also has a protective function. It is important to prevent placement of excessive weight on the affected limb that could lead to destruction of the inflamed laminar tissue. Therefore, a useful analgesic would control maladaptive pain (i.e., hyperalgesia, allodynia) while maintaining some degree of adaptive pain (i.e., pain that is protective to the organism). In the case reported here, the pain scores dropped sharply with the inclusion of *t*-TUCB, but as in rodents the sEHI did not abolish all nociceptive input from the feet. Modulation of hyperalgesia and allodynia with the use of sEHIs has been demonstrated in rodent models (Inceoglu et al. 2006; Schmelzer et al. 2006; Inceoglu et al. 2007; Inceoglu et al. 2008; Morisseau et al. 2010; Inceoglu et al. 2011; Wagner et al. 2011a; Wagner et al. 2011b). An analgesic that provides the above and is also able to arrest the progression of the disease would be highly desirable. In this context, pharmacologic inhibition of sEH fully prevented mortality in LPS-exposed mice by promoting inflammatory resolution as shown by reductions in plasma levels of pro-inflammatory cytokines and nitric oxide metabolites and increases in the synthesis of lipoxins (Schmelzer et al. 2005). As such, it is feasible that the improvement seen in this case of laminitis resulted from nociceptive modulation via several mechanisms, and possibly also from arresting of the inflammatory events in laminar tissue. Future studies are warranted to test this hypothesis.

Because laminitis is a complex disease we cannot distinguish the comparative contributions of the different known biological effects of sEHI. However, laminitis presents as laminar inflammation and inflammatory pain transitioning into chronic and possibly neuropathic pain (Hood 1999; Driessen et al. 2010). The association of hypertension could have a

number of causes including a response from pain itself. However, this complex disorder addresses the multiple advantages of sEH in reducing hypertension, inflammation, inflammatory pain, neuropathic pain and toxicity associated with NSAIDs and COXIBs (Node et al. 1999; Yu et al. 2000; Schmelzer et al. 2005; Inceoglu et al. 2006; Schmelzer et al. 2006; Chiamvimonvat et al. 2007; Inceoglu et al. 2007; Inceoglu et al. 2008; Imig & Hammock 2009; Revermann 2010; Wagner et al. 2011a).

No undesirable effects could be detected in the horse of this report. To date, no overt adverse effects associated with sEH inhibition have been observed in studies in rodents (Inceoglu et al. 2006; Schmelzer et al. 2006; Inceoglu et al. 2007; Inceoglu et al. 2008; Morisseau et al. 2010) dogs (Tsai et al. 2010), and non-human primates (Ulu et al. 2011) even when co-administered with NSAIDs (Schmelzer et al. 2006). In fact, sEH inhibitors appear to have a remarkable sparing effect in the analgesic action of NSAIDs (Schmelzer et al. 2006), suggesting that lower effective doses of NSAIDs could be used thus minimizing risk of side effects.

In conclusion, inhibition of sEH with *t*-TUCB was associated with a significant improvement in pain scores in one horse with laminitis whose pain was refractory to the standard of care therapy. No adverse effects were noticed. Future studies evaluating the analgesic and protective effects of these compounds in painful inflammatory diseases in animals are warranted.

Acknowledgments

This work was partially funded by NIEHS grant ES02710, NIEHS Superfund Basic Research Program grant P42 ES04699, and by the UC Davis Center for Equine Health.

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Figure 1.

Daily visual analog pain scores (**A**), systemic arterial blood pressure and heart rate (**B**) in one horse with pain due to laminitis, which was treated with multimodal analgesic therapy that included an investigational new drug inhibitor of soluble epoxide hydrolases (*t*-TUCB). The time frame of drug administration, along with doses, frequency and route of administration is presented above panel **A**. VAS= visual analog scale (0=no pain, 10= worst pain possible); SAP=systolic arterial pressure; MAP=mean arterial pressure; DAP=diastolic arterial pressure; HR=heart rate. BID=twice daily; SID=once daily; PO=orally; IV=intravenously.

Figure 2.

Plasma concentrations of the experimental drug inhibitor of soluble epoxide hydrolases *t*-TUCB (0.1 mg kg^{-1} SID, IV), phenylbutazone ($3\text{--}4 \text{ mg kg}^{-1}$ BID, PO) and gabapentin (20 mg kg^{-1} BID, PO) in one horse with pain due to laminitis.

Hematology and serum biochemistry values at baseline (before the first dose on day 8) and after the first (day 9), second (day 10) and fifth (day 13) dose of an experimental new drug inhibitor of soluble epoxide hydrolases (*r*-TUCB 0.1 mg kg⁻¹ IV) as part of multimodal analgesic therapy in one horse with pain due to laminitis.

Table 1

TEST	Day 8	Day 9	Day 10	Day 13	Reference limits
HEMATOLOGY					
Red Blood Cells (M μ L ⁻¹)	8.1	8.1	8.5	7.8	6.2–10.2
Hemoglobin (g dL ⁻¹)	13.1	13.1	13.6	12.5	11.2–17.2
Hematocrit (%)	33.5	33.1	34.9	32.1	30–46
Mcv (fL)	41.2	40.9	41.1	40.9	37–53
Mch (pg)	16.1	16.2	16	15.9	14–20
Mchc (g dL ⁻¹)	39.1	39.6	39	38.9	36–39
Rdw (%)	18.1	18	17.8	17.9	16–20
Anisocytosis	Slight	Slight	Slight	Slight	-
Echinocytes	-	Few	Few	Few	-
White Blood Cells (μ L)	9000	8170	11200	7320	5000–11600
Neutrophils (μ L)	5652	5245	8299	3967	3400–11900
Lymphocytes (μ L)	2943	2582	2498	3016	1600–5800
Monophils (μ L)	333	270	314	271	0–500
Eosinophils (μ L)	63	57	56	51	0–200
Basophils (μ L)	27	25	22	22	0–100
Platelets ($\times 10^3$ μ L ⁻¹)	250	231	257	251	100–225
Plasma protein (g dL ⁻¹)	7	7	6.9	6.8	5.8–8.7
Plasma fibrinogen (mg dL ⁻¹)	300	300	400	500	100–400
Pp:Pf	22	22	16	13	-
BIOCHEMISTRY					
Creatinine (mg dL ⁻¹)	1	1.1	1.1	1.1	0.9–2.0
Magnesium, Ionized (mmol L ⁻¹)	0.54	0.47	0.55	0.51	0.47–0.7
Anion Gap (mmol L ⁻¹)	12	12	11	12	9–17
Sodium (mmol L ⁻¹)	134	137	136	137	125–137
Potassium (mmol L ⁻¹)	4.3	3.8	4	3.8	3.0–5.6

TEST	Day 8	Day 9	Day 10	Day 13	Reference limits
Chloride (mmol L ⁻¹)	99	101	101	99	91–104
Bicarbonate (mmol L ⁻¹)	27	28	28	30	23–32
Phosphorus (mg dL ⁻¹)	3.3	3.7	2.7	3.4	2.1–4.7
Calcium (mg dL ⁻¹)	12.9	12.4	12.5	12	11.4–14.1
BUN (mg dL ⁻¹)	22	21	21	22	12–27
Glucose (mg dL ⁻¹)	104	88	104	97	50–107
Total Protein (g dL ⁻¹)	6.2	6.2	6.2	5.8	5.8–7.7
Albumin (g dL ⁻¹)	3.3	3.3	3.4	3.1	2.7–4.2
Globulin (g dL ⁻¹)	2.9	2.9	2.8	2.7	1.6–5.0
AST (IU L ⁻¹)	506	489	459	358	168–494
Creatine Kinase (IU L ⁻¹)	273	223	195	163	119–287
Alkaline Phosphatase (IU L ⁻¹)	118	119	126	113	86–285
GGT (IU L ⁻¹)	11	11	11	11	8–22
Triglycerides (mg dL ⁻¹)	24	32	30	35	2–41
Bilirubin Total (mg dL ⁻¹)	1.5	1.5	1.6	1.3	0.5–2.3
Bilirubin Direct (mg dL ⁻¹)	0.2	0.1	0.1	0.1	0.2–0.6
Bilirubin Indirect (mg dL ⁻¹)	1.3	1.4	1.5	1.2	1.7–3.6
SDH-37 (IU L ⁻¹)	0	0	0	0	0–8
Hemolysis Index	21	77	43	23	2
Icteric Index	3	3	3	2	-
Lipemic Index	5	5	6	9	-