Pharmacokinetics and antinociceptive effects of tramadol and its metabolite O-desmethyltramadol following intravenous administration in sheep

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Highligts

- Six sheep were administered 4 and 6 mg/kg tramadol and saline intravenously.
- Pharmacokinetics analysis and mechanical nociceptive threshold test were performed.
- Pharmacokinetics parameters of tramadol were similar after the two doses.
- No mechanical antinociceptive effects of tramadol were reported.
- Further studies are warranted to assess the efficacy of tramadol in sheep.

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Abstract

Although sheep are widely used as an experimental model for various surgical procedures there is a paucity of data on the pharmacokinetics and efficacy of analgesic drugs in this species. The aim of this study was to investigate the pharmacokinetics of intravenously (IV) administered tramadol and its active metabolite O-desmethyltramadol (M1) and to assess the mechanical antinociceptive effects in sheep.

In a prospective, randomized, blinded study, six healthy adult sheep were given 4 and 6 mg/kg tramadol and saline IV in a cross-over design with a 2-week wash-out period. At predetermined time points blood samples were collected and physiological parameters and mechanical nociceptive threshold (MNT) values recorded. The analytical determination of tramadol and M1 was performed using high performance liquid chromatography. Pharmacokinetic parameters fitted a two- and a non-compartmental model for tramadol and M1, respectively. Normally distributed data were analysed by a repeated mixed linear model.

Plasma concentration vs. time profiles of tramadol and M1 were similar after the two doses. Tramadol and M1 plasma levels decreased rapidly in the systemic circulation, with both undetectable after 6 h following drug administration. Physiological parameters did not differ

Introduction

Sheep are widely used as an experimental model for various surgical procedures (Coulter et al., 2009). In spite of this, there is a paucity of data regarding the pharmacokinetics and efficacy of analgesic drugs in this species. There is a clear need to identify analgesic drugs, dose and dose interval for use in sheep during invasive experimental procedures.

Tramadol is an analgesic drug widely used in people and in small animals; it possesses a weak agonist action against the mu (μ) opioid receptor and inhibits the reuptake of norepinephrine and serotonin (Raffa et al., 1992). The active metabolite, O-desmethyltramadol (M1) has an affinity for the μ opioid receptor that is 300× greater than that of tramadol (Grond and Sablotzky, 2004). No studies investigating the analgesic efficacy of tramadol in sheep have been performed so far. However, the pharmacokinetics and biotransformation of tramadol have been studied in several animal species including the dog, cat, goat, llama, alpaca, horse and donkey (KuKanich and Papich, 2004; Giorgi et al., 2007, 2009a; de Sousa et al., 2008; Pypendop and Ilkiw, 2008; Cox et al., 2011; Stewart et al., 2011; Edmondson et al., 2012), highlighting species-specific differences in the kinetic profiles of both the parent drug and its metabolites.

Although the effectiveness of tramadol is still unclear in veterinary medicine (Giorgi, 2012), there are reports confirming the analgesic efficacy of tramadol for the management of peri-operative pain in other ruminants (Bigham et al., 2010; Habibian et al., 2011; Dehkordi et al., 2012).

To evaluate the analgesic or antihyperalgesic efficacy of opioid drugs, nociceptive threshold testing, or analgesiometry, can be used. This consists of the application of a measurable stimulus, usually mechanical, thermal or electrical, in order to obtain a clear behavioural response and record the threshold at which the animal responded. If the tested drug exerts analgesic or antihyperalgesic effect, the threshold will either increase or remain unchanged (for example, when thresholds are

measured following induction of inflammation). Mechanical nociceptive threshold (MNT) testing devices have already been tested and validated in sheep (Nolan et al., 1987a; Musk et al., 2014).

The aim of the present study was to investigate the pharmacokinetic profile and antinociceptive efficacy of two different doses of tramadol administered intravenously (IV) to sheep.

Materials and methods

Animals and treatments

Six female adult Brogna sheep, body mass between 38 and 55 kg, were enrolled in the study, which was performed with approval from the Ethical Committee for Animal Experimentation of the University of Padua (CEASA 80/2012, 30 April 2013) and according to EC Council Directive 86/609EEC (Council of the European Communities, 1986).

All animals were considered healthy based on clinical examination and haematological analyses. Sheep were kept indoors in a group pen (400 × 400 cm) in the Large Animal Facility at the University of Padua and fed a commercial pellet and hay diet. On the day of the experiment, three sheep were moved into individual stalls where the animals remained in visual contact with each other. The dimensions of each pen were: length 160 cm, width 66 cm and height 110 cm. Pens were bedded with straw. Sheep were acclimatized to the stalls, handlers, the MNT probe and testing procedure prior to commencing the study. Sheep were deprived of food for 8 h prior to the start of the experiment while water was available ad libitum. Hay and water were available ad libitum 2 h after treatment administration.

Two 14G catheters (Delta Ven, DeltaMed) were placed in the right and left jugular veins, to allow both treatment administration and collection of blood for the pharmacokinetic analysis. All

six sheep received the following three treatments IV over 2 min via the left jugular catheter: (1) tramadol 4 mg/kg (Group T4) (Tramadolo Hexal Ag), (2) tramadol 6 mg/kg (Group T6), and (3) 5 mL of sodium chloride 0.9% solution (Group SAL). Drugs were administered in a randomly allocated, crossover design with a 2-week wash out period between treatments. Investigators were blinded to treatment allocation.

Blood sampling and clinical evaluation

Five millilitres of blood were collected from the right jugular vein before drug (or saline) administration, 5, 10, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 12 and 24 h after administration. Whole blood was placed in lithium-heparinized tubes and centrifuged at 2000 *g* for 5 min. The harvested plasma was frozen at -80 °C until pharmacokinetic analysis was performed.

Immediately before and 15, 30 min and 1, 1.5, 2, 4, 6, 8, 10 and 12 h after drug administration, heart and respiratory rates were determined by thoracic auscultation and observation of thoracic excursions respectively. Rectal temperature and reticulo-ruminal motility, assessed by auscultation of the rumen (number of cycles in 5 min), were monitored starting from 30 min after drug administration. Sedation was quantified using a 0-100 mm visual analogue scale (VAS) scale where 0 mm was considered no sedation and 100 was considered very deep sedation/unconsciousness. Any adverse events attributed to the drug treatment were noted throughout the course of the study.

MNT Testing

MNT was measured by a single investigator using the ProdPro (Topcat Metrology), as described elsewhere (Dixon et al., 2010). Briefly, this mechanical testing device comprises a cuff with a 2 mm hemispheric blunt pin fixed on a rolling diaphragm actuator and is applied perpendicular to the skin of the test area, in this case the dorsal aspect of the right metacarpus

approximately 4 cm below the carpus. The pin was pushed against the skin with a force which was applied manually by a syringe, connected to non-distensible tubing via a digital meter which displayed the force exerted, until a clear withdrawal response (leg lift, head turn, weight bearing on the contra-lateral limb) was evoked. The force at which the sheep responded with a clear withdrawal response was recorded as the MNT. A dummy actuator, identical to the test actuator apart from the fact that it did not contain the pin was secured to the contra-lateral limb. A cut off point was set at 25 N in order to prevent tissue trauma should a clear withdrawal response not be elicited.

The MNT was measured prior to blood collection at time point 0, immediately before drug administration (baseline), 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10 and 12 h after drug administration. In order to calculate the MNT, three measurements were performed at each time point with an interval of at least 2 min between each measurement and the mean was used for statistical analysis; five tests were performed and averaged to obtain the baseline MNT.

Tramadol and M1 determination in blood

Based on a previously published high performance liquid chromatography (HPLC) technique (Giorgi et al., 2009b), the analytical method was briefly re-validated in sheep plasma. The HPLC was a liquid chromatographic system (Jasco) consisting of high-pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus) and a 20 μL loop. Data were processed by Borwin software (Jasco). Chromatographic separation assay was performed by a Luna C18 ODS2 analytical column (150 × 4.6 mm inner diameter, 3 μm particle size, Phenomenex) maintained at 25 °C. The mobile phase consisted of acetonitrile:buffer (20 mM sodium dihydrogen phosphate, 30 mM sodium dodecyl sulfate, and 15 mM triethylamine, adjusted to pH 3.9 with phosphoric acid) (40:60 V/V) at a flow rate of 0.8 mL/min. Excitation and emission wavelengths were 275 and 300 nm, respectively. The analytical method used in this study was able to

differentiate the three main metabolites (M1, M2 and M5). However, the M2 and M5 plasma concentrations are not presented here as they are inactive metabolites and hence of negligible importance for the study.

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated for each subject from tramadol and M1 plasma concentrations vs. time curves using WinNonLin v 5.3 (Pharsight Corp). The comparison between competing models (one- vs. two-compartment) was made using the Akaike test. The best fit was described by a two-compartment open and a non-compartmental model, for tramadol and M1, respectively. The area under the concentration vs. time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule (Gibaldi and Perrier, 1982).

Statistical analysis

Sample size calculations were performed before commencing the study. For a two way repeated measures ANOVA with a difference between Δ MNT means (Δ MNT= MNT value at a specific time point minus baseline MNT value) of 3.5 N, standard deviation (SD) =2, $\beta = 0.8$ and $\alpha = 0.05$, a minimum of 6 animals per group were required. Residuals of repeated measures for Δ MNT, heart rate, respiratory rate, body temperature were analysed for normality using the Shapiro-Wilk test.

Normally distributed data were analysed by a repeated mixed linear model with the fixed effects of treatment, time and their interaction and animal as a random effect (Littell et al., 1998). Reticulo-ruminal motility was analysed by a nonparametric approach (Kruskal-Wallis) to test the effect of treatment at the different time points. Data analyses were performed using SAS statistical software (version 9.3, SAS Institute). *P* values < 0.05 were deemed significant.

Results

Pharmacokinetics

The tramadol and M1 concentrations vs. time after IV administration of 4 and 6 mg/kg of tramadol are shown in Fig. 1. The limits of detection (LOD) were 1 ng/mL and 3 ng/mL and the limits of quantification (LOQ) were 5 ng/mL and 10 ng/mL for T and M1, respectively. The values of precision for both analytes were always ≤ 9.8 (CV%), while accuracy was < 7.3%.

At the first time point (5 min) the plasma concentrations of tramadol were 1.29 ± 0.17 µg/mL and 1.56 ± 0.10 µg/mL following treatment with 4 mg/kg and 6 mg/kg tramadol, respectively. At the subsequent time points, tramadol plasma concentrations decreased rapidly for both treatments and were detectable in all animals only up to 4 h post-administration. At 6 h, tramadol was detectable in 5/6 sheep after treatment with 6 mg/kg and following administration of 4 mg/kg, was detectable at this time point in 4/6 animals. M1 was detectable in the plasma 5 min after tramadol administration, with a concentration equal to 0.13 ± 0.02 and 0.14 ± 0.03 µg/mL after administration of 4 and 6 mg/kg of tramadol, respectively. Similar plasma concentrations were maintained up to 45 min and then plasma concentrations decreased over the next 4 h. At time points later than 4 h, plasma concentrations of M1 were < LOQ. The most important pharmacokinetic parameters of tramadol and M1 are reported in Tables 1 and 2, respectively.

Clinical evaluations

Mild self-limiting adverse events were noticed in all animals in Group T6 and in four animals in Group T4. These included tremors, muscle fasciculation, ataxia, agitation, urination and defecation that started 15-30 s after the beginning of drug administration and lasted for a maximum of 10 min. The severity of adverse events was greater in Group T6 but in all cases they spontaneously resolved. No adverse events were recorded in Group SAL. Heart rate, respiratory rate, temperature and reticulo-ruminal motility were not statistically different within each group

compared to baseline values or between groups at any time points ($P > 0.05$). No sedation was observed during the experiment in any group (VAS = 0 mm).

MNT testing

Animals reacted to the MNT stimulation with a leg lift or head turn. The cut off value of 25 N was never reached during the study and no signs of tissue trauma or lameness were observed in sheep. There were no significant differences between groups in MNT baseline values; the overall baseline MNT was 8 ± 1.9 N.

There were no differences in Δ MNT between groups at any time point ($P > 0.05$). Independently from treatment, at 15 and 30 min post-administration the Δ MNT values were significantly higher than those observed from the 360 min time point onwards ($P < 0.001$). Δ MNT values are shown in Fig. 2. Within-group comparisons showed that there were no statistically significant differences between the basal MNT and the MNT at any different time point ($P > 0.05$).

Discussion

Sheep are widely used for invasive biomedical research but there are limited data on analgesic drug administration in this species. Few analgesic drugs have marketing authorisations for use in ruminants but those that are available include non-steroidal anti-inflammatory drugs (NSAIDs), α_2-agonists and local anaesthetic agents. In people, tramadol provides good analgesia with only mild effects on cardio-respiratory function and intestinal motility (Raffa et al., 1992) and is not currently subject to Controlled Drug legislation in Europe.

The tramadol doses chosen in the present study were extrapolated from previous studies in other ruminant species (de Sousa et al., 2008; Cox et al., 2011; Edmondson et al., 2012). A

239 pharmacokinetic study in goats evaluated 2 mg/kg tramadol (de Sousa et al., 2008) and the resulting
240 data suggested that 4 mg/kg would be an appropriate dose to achieve plasma concentrations that
241 might be consistent with analgesia, although antinociceptive/analgesic efficacy was not measured
242 concurrently in that study.
243
244 The plasma concentration vs. time profiles (Fig. 1) of tramadol and M1 were similar after the
245 two doses. Blood concentrations of tramadol in sheep declined quickly as evidenced by the very
246 short half-life and high clearance value after administration of 4 and 6 mg/kg. The elimination half-
247 life values in this study were lower than those observed in other species such as goats (0.94 h) (de
248 Sousa et al., 2008), alpacas (0.78-0.85 h) (Giorgi et al., 2010; Edmondson et al., 2012), and llamas
249 (2.12 h) (Cox et al., 2011).
250
251 The formation of the active metabolite M1 was observed in all sheep. This is in agreement
252 with an earlier study in goats (de Sousa et al., 2008), while in alpacas (Giorgi et al., 2010) M1 was
253 detected in only 1/8 treated animals. In our study, the ratio of AUCs for M1/T was equal to 0.36 and
254 0.43 after IV administration of 4 mg/kg and 6 mg/kg of tramadol, respectively. These similar values
255 suggest that the metabolic system of the sheep was not saturated at doses up to 6 mg/kg. This ratio
256 value is similar to that found in dogs (0.31) by KuKanich and Papich (2004), and in goats (0.28) by
257 de Sousa et al. (2008), and lower than that observed in llamas (0.94) by Cox et al. (2011) and in cats
258 (AUCs ratio M1/T >1) by Pypendop and Ilkiw (2008). These comparisons indicate that M1 has a
259 more prominent role in the pharmacokinetics of tramadol in cats and llamas compared to sheep.
260
261 In people, the minimum effective concentrations reported for tramadol and M1 are 0.3 ± 0.2
262 µg/mL (Lehmann et al., 1990) and 0.08 ± 0.03 µg/mL (Grond et al., 1999), respectively. In our
263 study, tramadol in plasma was above the human therapeutic concentration up to 45 min after drug
264 administration while the M1 plasma concentrations considered effective in people were maintained

in sheep plasma up to 2 h post treatment. Surprisingly, we found no mechanical antinociceptive effect of tramadol in the first hour after drug administration, when plasma levels of tramadol and M1 were similar to analgesic concentrations reported in humans.

Quantitative sensory testing methods have been used in conscious painful and non-painful/healthy sheep in order to assess the efficacy of analgesic drugs, including opioids (Nolan et al., 1988; Waterman et al., 1991; Kyles et al., 1993; Musk et al., 2014), NSAIDs (Welsh and Nolan, 1994,1995; Lizarraga and Chambers, 2006) and α_2-agonists (Grant et al., 2001; Grant and Upton, 2004; Musk et al., 2014). We found no statistically significant difference in MNT between groups which is consistent with other studies performed in conscious healthy sheep. Buprenorphine (6 µg/kg IV) was found to exert antinociceptive activity in a thermal nociceptive threshold test but not in the mechanical one (Nolan et al., 1987b); butorphanol (0.1-0.4 mg/kg IV) did not cause any significant elevation in mechanical pressure threshold (Waterman et al., 1991); pethidine (5 mg/kg IV) increased thermal threshold for 30 min but pressure threshold only for a few minutes (Nolan et al., 1988) and pethidine plus fentanyl caused a brief increase in mechanical threshold values (Nolan et al., 1987a).

Clearly a more complete evaluation of analgesic effects of a drug should be performed using more than one type of stimulus (Tyers, 1980). Thermal nociceptive threshold testing was not performed in this study because of the unavailability of the equipment and for economic reasons, but also because it has been reported to cause skin damage in sheep (Musk et al., 2014), most likely because of the stoical attitude of this species. Moreover, when tramadol was tested in conscious horses at the dose of 2 mg/kg, no changes were detected with a thermal nociceptive threshold model (Dhanjal et al., 2009).

The lack of efficacy of tramadol observed in the present study may be due to several reasons. It might be that the achieved plasma concentrations of tramadol were not sufficient to promote antinociception in sheep and that higher plasma concentrations would be required. Genetic variabilities were shown to affect tramadol metabolism in people (Pedersen et al., 2006) and this may also apply to sheep. A variation in the analgesic effect of xylazine in different breeds of sheep has been reported (Ley et al., 1990). Moreover, sheep tend to mask signs of nociception, although in the current study very clear behavioural end points to the MNT test were produced and the sheep did not reach the cut-out values. Xylazine, which has been shown to cause an increase in the mechanical nociceptive threshold in sheep (Nolan et al., 1987c), was not used as a positive control as it would have increased the mechanical nociceptive threshold but it would be difficult to differentiate between sedation and analgesia.

It should be noted that a major limitation of nociceptive threshold testing is that it does not provide the same stimulus as clinical pain (Love et al., 2011). It may be possible that the analgesic effects of tramadol would be detected in clinical pain states.

The MNT decreased with time in all groups, which might be explained by a sensitization to the MNT test. This finding is consistent with previous reports of MNT measurement in sheep (Stubsjoen et al., 2010) and could be another reason why no analgesic effect of tramadol was detected. On the other hand, in another report the mechanical nociceptive threshold did not vary over 14 days in conscious healthy sheep (Abu-Serriah et al., 2007). In our study, in order to prevent bias, the same observer performed the MNT test and animals were acclimatised to research personnel, equipment, procedures and stables.

After tramadol administration, adverse events, including muscle fasciculation, tremors, agitation and ataxia, were noticed in the majority of animals, but these were short lasting and self-

316 limiting and not deemed to be clinically problematic. This is consistent with findings described in
317 alpacas (Giorgi et al., 2010; Edmondson et al., 2012), llamas (Cox et al., 2011), and horses (Giorgi
318 et al., 2007; Stewart et al, 2011). Although drugs were injected over 2 min, adverse events were still
319 observed. In people, dose and speed of infusion of tramadol affect the incidence of adverse events
320 (Grond and Sablotzki, 2004). In the clinical setting in sheep, a slow infusion rate, over 10 min, may
321 produce less adverse effects.
322
323 Compared to saline, tramadol administration did not affect measured physiological parameters
324 including heart rate, respiratory rate and rectal temperature. Other authors have also observed an
325 absence of change in these parameters after epidural administration of tramadol in goats and cows
326 (Bigham et al., 2010; Dehkordi et al., 2012). In contrast, a study conducted in lambs has shown
327 changes in rectal temperature and heart and respiratory rate (Habibian et al., 2011). These
328 incongruities might be the result of having adult versus juvenile subjects and differences in route of
329 administration. In our work tramadol was shown not to affect gut motility; this might be due to the
330 low affinity of tramadol for the μ-opioid receptor and thus tramadol may be advantageous in this
331 species. Tramadol administered to horses at the dose of 2 mg/kg IV was shown not to alter the
332 faecal output although a short lived (40 min) decrease in borborygmus score was reported (Dhanjal
333 et al., 2009). Further studies could be performed to assess the effect of tramadol on gastrointestinal
334 motility by quantification of faecal output (Love et al., 2012) or using radiopaque spheres (Sano et
335 al., 2011).
336
337 **Conclusions**
338 IV administration of tramadol at 4 and 6 mg/kg in sheep was associated with rapid
339 metabolism and a transient presence of M1 in plasma; antinociceptive effects were not detected
340 using an MNT model. This study provided pharmacokinetic data for tramadol in sheep but further
341 studies are warranted to assess its clinical efficacy in animals experiencing pain.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper. Dr P.M. Taylor and Dr M.J. Dixon, from Topcat Metrology Ltd, UK, gave some advice regarding the study design but played no role in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication.

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Figure legends

Fig. 1. Average tramadol (solid line, triangle) (-▲-) and M1 (dotted line, square) (-■-) concentrations vs. time after IV administration of tramadol 4 mg/kg (a) and 6 mg/kg (b) (*n* = 6), respectively. Bars represent the standard deviation.

Fig. 2. ΔMNT values at the different time points in the three groups of sheep (*n* = 6). Saline = grey; T4 = light grey; T6= dark grey. Bars represent the standard deviation.

Table 1

Main average pharmacokinetic parameters of tramadol following tramadol IV administration at 4 mg/kg and 6 mg/kg in sheep (*n* = 6)

Dose		4 mg/kg		6 mg/kg	
Parameter	Unit	Mean	SD	Mean	SD
k_{10}	1/h	6.895	7.350	2.210	0.381
k_{12}	1/h	7.652	10.137	1.658	1.188
k_{21}	1/h	3.102	1.243	3.062	1.269
$t_{1/2\alpha}$	h	0.091	0.078	0.161	0.118
$t_{1/2\beta}$	h	0.671	0.419	0.573	0.116
V_1	L/kg	1.572	1.151	2.870	0.120
CL_1	L/kg/h	4.862	1.191	6.315	0.949
V_2	L/kg	1.694	0.890	1.415	0.796
CL_2	L/kg/h	4.466	1.473	4.732	3.509
$AUC_{0-\infty}$	μg/mL*h	0.870	0.236	0.968	0.145
AUMC	μg/mL*h^2	0.539	0.245	0.671	0.215
MRT	h	0.651	0.337	0.686	0.137
V_{ss}	L/kg	3.266	1.919	4.285	0.745

AUC $_{0-\infty}$, area under serum concentration-time curve from time zero to infinity; AUMC, area under moment curve; CL_1, clearance of central compartment; CL_2, clearance of peripheral compartment; k_{10}, the rate at which the drug leaves the system from the central compartment (the elimination rate); k_{12}, the rate at which the drug passes from central to peripheral compartment; k_{21}, the rate at which the drug passes from peripheral to central compartment; MRT, mean residence time; $t_{½\alpha}$, distribution half-time; $t_{½\beta}$, elimination half-time; V_1, volume of distribution in central compartment; V_2, volume of distribution in peripheral compartment; V_{ss}, volume of distribution at steady state.

SD, standard deviation.

Table 2

Average pharmacokinetic parameters of M1 following tramadol IV administration at 4 mg/kg and 6 mg/kg in sheep (*n* = 6)

Dose		4 mg/kg		6 mg/kg	
Parameter	Unit	Mean	SD	Mean	SD
λz	1/h	0.606	0.084	0.580	0.142
$t_{½\lambda z}$	h	1.163	0.163	1.266	0.350
$T_{max\ obs}$	h	0.373	0.334	0.402	0.267
$C_{max\ obs}$	µg/mL	0.141	0.020	0.159	0.037
$AUC_{0-\infty\ obs}$	µg/mL*h	0.317	0.077	0.414	0.128
$MRT_{0-\infty\ obs}$	h	1.810	0.244	1.974	0.388

AUC $_{0-\infty\ obs}$, area under serum concentration-time curve from time zero to infinity; $C_{max\ obs}$, Maximum concentration observed; MRT $_{0-\infty\ obs}$, mean residence time from time zero to infinity;

548 $T_{max\ obs}$, Time of maximum concentration observed; $t_{½\lambda z}$,

549 terminal half-time.

550 SD, standard deviation.

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