Comparison of the hypothalamic–pituitary–adrenal axis susceptibility upon single-dose i.m. depot versus long-acting i.v. triamcinolone acetonide therapy: a direct pharmacokinetic correlation

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Abstract
The effects of single injections of glucocorticoid (GC) depot suspension and of long-acting GC were studied in conscious dogs. Both the depot suspension GC triamcinolone-16,17-α-acetonide (TAA) and the long-acting triamcinolone acetonide-21-dihydrogen phosphate (TAA-DHP) decreased basal and ACTH-stimulated cortisol levels and in a specific time-dependent way. Before treatment, all dogs had normal basal and peak cortisol responses to ACTH challenge (13–15 and >120 nmol/l at 1 h respectively). Intravenous TAA-DHP reduced cortisol levels for 12 h, i.m. TAA reduced cortisol levels as of 1.5 h and the effect lasted for at least 4 weeks. Both treatments blunted the peak response to ACTH. ACTH elevated cortisol levels to or above baseline values within 10 days following TAA-DHP treatment, but the TAA treatment suppressed an ACTH response for at least 4 weeks. Kinetic analysis of both the preparations demonstrated rapid absorption (t_{max}, 0.6–1.5 h) and low maximum plasma concentrations (peak C_{max}, 2.99–5.51 nmol/l) of the steroids; indeed, the terminal half-life of TAA-DHP (13.9 ± 1.3 h) was very much shorter than that of TAA (125.9 ± 15.8 h). In addition, the mean residence time differed very much (11 vs 160 h for TAA-DHP and TAA respectively), in line with a delayed elimination of the depot compared with the long-acting formulation. Application of these TAA formulations needs careful evaluation for their surprisingly different effects on endocrine stress axis activity.

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Introduction
Triamcinolone acetonide (TAA), a synthetic glucocorticoid (GC; 9α-fluoro-16α-hydroxyprednisolone), is a more potent intermediate-acting derivative of triamcinolone and has eight times more potency than prednisone. The intravenously used long-acting TAA is available as water-soluble phosphate ester, e.g. triamcinolone acetonide-21-dihydrogen phosphate (TAA-DHP), which is supposed to be hydrolysed rapidly in the body to form the free corticoid alcohol (Möllmann et al. 1985). The depot i.m. formulation of triamcinolone acetonide, e.g. triamcinolone-16,17-α-acetonide (TAA), is available as a sterile crystalline suspension. This formulation is practically insoluble in water, and hence provides a depot effect with constant release of the active agent from the injection site over a long period of time.

In general, GCs are potent anti-inflammatory drugs used for the treatment of a variety of disorders. When given systemically, they are known to induce many side effects. To avoid multiple dosing of short-acting GCs, long-acting GCs, including TAA are preferred formulations for the treatment of several autoimmune diseases, such as in systemic lupus erythematosus and multiple sclerosis and/or serious inflammatory diseases as well as in diabetic retinopathy (Scher 1964, Heun et al. 1992, Doggrell 2001, Jonas et al. 2001, Massin et al. 2004, Hoffmann et al. 2006). Moreover, depot formulation of injectable TAA is effective in GC-resistant asthma which is poorly controlled by β2-adrenoceptor agonists (Doggrell 2001). The most common and best studied use of this drug formulation is for the treatment of joint disorders (Roberts et al. 1996, Kumar et al. 2004) and several exudative diseases of the eye (Gillies et al. 2003, Jonas et al. 2003).

Suppression of the hypothalamic–pituitary–adrenal (HPA) axis function has been well documented as one of the most important potential complications upon repeated systemic GC therapy (Tuft et al. 1971, Miyamoto et al. 1972, Mikhail et al. 1973, Dallman et al. 1994), a paradigm based in part upon administration of high non-physiological doses of GCs (Baxter 2000, Zaloga & Marik 2001). This drawbacks is associated with decreased production of the hypothalamic corticotrophin-releasing hormone (CRH) and the pituitary adrenocorticotrophic hormone (ACTH; Keller-Wood & Dallman 1984, Antoni 1986, Dallman et al. 1987). Many studies have demonstrated that repeated and high dosing treatment with short- or long-acting GCs adversely affect the vulnerability of the HPA axis activity and adrenal function with a consequent decrease in plasma cortisol levels. Only a few studies addressed single-dose depot TAA administration and the risk to induce long-term adverse effects. Recently,

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a case of acute iatrogenic Cushing's syndrome and HPA axis suppression which persisted 8 months has been reported in humans after a single-dose i.m. depot TAA administration (Iglesias et al. 2005). However, in another study on dogs, the adrenocortical responsiveness recovered within 2 weeks after i.m. TAA injection (Kempainen et al. 1982). Such delayed HPA axis and adrenal responses after the single-dose depot, TAA seem to be associated with the delayed pharmacokinetics of the drug, but direct evidence is lacking so far. Few studies have focussed merely on the pharmacokinetics of the long-acting TAA and its water-soluble phosphate ester (TAA-DHP) after i.v. administration, and showed that TAA exhibits a high clearance, high volume of distribution and short half-life (Möllmann et al. 1985, Derendorf et al. 1995, Rohatagi et al. 1995).

Since repeated administration of GCs eventually suppresses HPA axis activity, we were interested to compare two long-acting GC formulations for their effect on HPA axis activity. We have determined the time dependence of the effects of TAA and TAA-DHP treatment (single injections) on basal and ACTH-stimulated cortisol levels and assessed the pharmacokinetics of the TAA formulations.

Materials and Methods

Chemicals

Triamcinolone acetonide-21-dihydrogen phosphate (TAA-DHP; volon A soluble 40 mg) was purchased from Mibe GmbH Arzneimittel (Brehna, Germany), and the depot formulation of TAA was a kind gift of AniMedica GmbH (Senden-Bössensell, Germany). Triamcinolone acetonide was purchased from Sigma. The ELISA Kit was obtained from Bio-X Diagnostics (Jemelle, Belgium). 3H-cortisol (specific activity, 50–90 Ci/mmol) as recently described (Abraham et al. 2005). The detection limit was 0.3 nmol/l; coefficient of variations were 8-1% (intra-assay) and 10-2% (interassay; N=6) at plasma cortisol levels measured in the animals. All samples were obtained after centrifugation of the whole blood (4000 g, 10 min, 4°C), and the aliquots were stored at −20°C until analysis.

Assay of cortisol

Cortisol was assayed by RIA using commercially available 3H-cortisol (specific activity, 50–90 Ci/mmol) as recently described (Abraham et al. 2005). The detection limit was 0.3 nmol/l; coefficient of variations were 8-1% (intra-assay) and 10-2% (interassay; N=6) at plasma cortisol levels measured in the animals. All samples were obtained after centrifugation of the whole blood (4000 g, 10 min, 4°C), and the aliquots were stored at −20°C until analysis.

Experimental design

All animals were investigated on two consecutive occasions with a 2-week washout period in a single-blind, crossover and random order. First, animals received a single dose of the long-acting formulation TAA-DHP (0.2 mg/kg) intravenously; the response of basal cortisol was measured in blood plasma samples taken at 0 h (control and baseline) and at 0, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 36, 48, 72 and 96 h, and at days 8 and 10 after injection. The ACTH-stimulated cortisol response at this occasion was determined on 0 h (baseline) as well on 48 h and day 10 after treatment. Secondly, 2 weeks after TAA-DHP when the animals had recovered as indicated by normalised plasma cortisol levels, the animals received the TAA formulation (i.m.). Sampling was at 0, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 36, 48, 72 and 96 h, and at days 8, 10, 15, 17, 22 and 29, to cover a full 4-week period. ACTH-stimulated cortisol levels were assessed at 0 h (baseline) and 48 h, and at days 10 and 29. All tests and blood sampling were started between 0800 and 1000 h. The dogs were fasted overnight before experiments.

HPA axis activity was tested by a bolus injection of synthetic ACTH (synacthen, 250 µg per animal, not corrected for body weight) at 0800 h and blood was taken 1 h after this ACTH challenge. For the analysis of basal and ACTH-stimulated cortisol levels as well as for the pharmacokinetic analysis of both substances, blood samples were usually collected at 0800 h from the cephalic vein by venipuncture in EDTA-containing tubes (Sarstedt, Hamburg, Germany). Plasma samples were obtained after centrifugation of the whole blood (4000 g, 10 min, 4°C), and the aliquots were stored at −20°C until analysis.

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removed for liquid scintillation analysis (Perkin–Elmer Life Sciences, Freiburg, Germany). Sample cortisol was estimated from comparison with standards run in triplicate; cortisol concentrations are expressed in nanomolar per litre. Samples from individual dogs were always run in the same assay.

Assay of TAA

Plasma concentrations of TAA were measured using a commercially available enzyme immunoassay (ELISA). This kit is designed for screening of TAA in milk, urine, tissue and feed samples. A calibration line was generated for each analysis by assaying TAA standards (0.02–0.46 nmol/l). The limit of quantification (LOQ) was calculated in 18 representative blank samples (plasma samples without TAA). The LOQ was calculated as the apparent content corresponding to the value of the mean plus three times the S.E.M. for the blank determinations. The LOQ was 0.14 nmol/l plasma. The coefficient of variance for the interassay precision was <11%.

The assay was carried out according to the manufacturer’s instruction. In brief, plasma (100 μl) was extracted with 4 ml diethyl ether after adding 400 μl distilled water. After agitation of the samples for 20 min at room temperature, they were centrifuged at 1000 g for 10 min. The organic phase was then evaporated to dryness, and each sample was dissolved in 1 ml phosphate buffer (34 mM, pH 7.4). Samples (50 μl) were added to each sheep anti-rabbit IgG-coated microplate wells (96-well plate) with 100 μl conjugate solution (TAA plus peroxidase) and 100 μl anti-TAA antibody solution. Then the wells were incubated for 2 h at 4 °C. After washing the well plates, 150 μl substrate (tetramethylbenzidine) were added to each well, and incubation was carried out light protected for 30 min at room temperature for colour development. The reaction was terminated by adding 50 μl sulphuric acid (3 M) and the optical density for each well was determined at 450 nm (TECAN Deutchland GmbH, Crailsheim, Germany; Spectra Thermo). Blank plasma and a positive control were included in each strip when analysing samples.

Pharmacokinetic data for plasma concentrations of TAA in each animal were analysed using least-squares non-linear regression analysis (Heinzel et al. 1993). The calculated highest plasma concentration of TAA was designated $C_{\text{max}}$ and the time this value was reached designated $T_{\text{max}}$. The area under the curve (AUC) was calculated using the trapezoidal rule. The total body clearance (CL) was calculated as dose divided by AUC. Mean residence time (MRT) was determined as area under the first moment curve divided by AUC.

Statistical analysis

Data presented in figures and tables are means ± S.E.M. ($N=10$). Statistical analysis of the data obtained with the RIA (basal and ACTH-induced cortisol levels) was performed using GraphPad prism software (version 4.0; GraphPad Software, San Diego, CA, USA). Statistical significance of differences was analysed by unpaired two-tailed Student’s $t$-test or, if appropriate, by repeated measures ANOVA followed by $t$-test using Bonferroni corrections for multiple comparisons. A $P$ value $<0.05$ was taken to indicate significance of differences. The statistical evaluation was performed using GraphPad prism software as mentioned earlier.

Results

Basal and ACTH-stimulated cortisol levels

Figure 1A and B shows the results of in vivo experiments from two consecutive occasions, i.e. after a single i.v. and i.m. injection of TAA-DHP and TAA respectively. A significant interaction between time and treatment ($P<0.01$) for the plasma cortisol was observed. One and a half hours after
TAA-DHP injection, basal cortisol had decreased below baseline values (14·35 ± 2·60 vs 6·67 ± 0·94 nmol/l; P < 0·05; Fig. 1A). The largest response (inhibition of basal cortisol) following TAA-DHP was observed at 8 h following injection (1·73 ± 0·94 nmol/l; P < 0·01). Even though basal cortisol had recovered after 24 h, the ACTH response was still blunted at 48 h after the injection of TAA-DHP (Fig. 1A, inset; 62·37 ± 4·01 vs 123·66 ± 4·15 nmol/l; P < 0·01 vs time 0), but reached baseline values after 10 days.

In contrast, as shown in Fig. 1B, the depot i.m. GC formulation (TAA) declined significantly (P < 0·01) the baseline (0 h) cortisol concentrations (13·94 ± 1·01) until day 15. The lowest cortisol concentrations were steady between 4 h and day 8 after injection. Here, the cortisol rise towards the baseline values was markedly delayed when compared with TAA-DHP, and baseline values were achieved only after about 3 weeks (day 22). On average, after the injection of 250 μg ACTH, maximum cortisol concentrations had decreased by approximately 58, 64 and 37% respectively, below values of pre-treatment stimulation after 48 h, 10 and 29 days of single TAA dose (Fig. 1B, inset; P < 0·01 vs time 0); this formulation consistently delayed the HPA axis recovery.

Gender did not affect basal cortisol levels (males 14·76 ± 2·26 nmol/l, n = 4 vs females 13·39 ± 0·89 nmol/l, n = 6) and cortisol responses after ACTH challenge (males 126·02 ± 8·01 nmol/l, n = 4 vs females 122·09 ± 4·96 nmol/l, n = 6).

**Plasma TAA following TAA and TAA-DHP injection**

The mean plasma concentration (± S.E.M.) of TAA measured over 36 h after i.v. administration of a single-dose TAA-DHP (0·2 mg/kg) indicated rapid decline of the GC reaching values at or below the limit of quantification after 8 h. Figure 2A depicts the mean plasma concentration–time profile after i.v. administration. The mean pharmacokinetic parameters calculated by non-compartmental methods are given in Table 1. The tmax of TAA after a single i.v. dose occurred at 0·6 ± 0·1 h, reaching a Cmax value of 5·8 ± 0·2 nmol/l. The total body clearance was 0·5 ± 0·1 ml/min per kg. The terminal half-life was 13·9 ± 1·3 h, and the MRT 10·7 ± 1·3 h, indicating a relatively fast elimination of the active substance. The volume of distribution at steady-state level was 0·6 ± 0·1/l/kg.

The mean plasma concentrations of TAA after i.m. depot single dose (0·2 mg/kg) are depicted in Fig. 2B, and the mean pharmacokinetic data are listed in Table 1. At tmax 1·5 ± 0·1 h, the maximum plasma concentrations (Cmax) of TAA were 3·1 ± 0·3 nmol/l, amounting about 50% of the initial concentration observed after i.v. administration (5·8 ± 0·2 nmol/l; peak levels of TAA occurred 30 min after i.v. injection). Subsequently, TAA levels decreased, but were still detectable 29 days after a single-dose i.m. depot injection. The area under the plasma concentration curve (AUC) was larger than the AUC after i.v. TAA-DHP injection (134·2 ± 8·4 vs 17·9 ± 1·7 nmol/h per l). The total body clearance for the depot formulation was 0·1 ml/min per kg. The terminal half-life for the depot TAA was 1·3 h, and the MRT 10·7 ± 1·3 h, indicating a relatively fast elimination of the active substance. The volume of distribution at steady-state level was 0·6 ± 0·1/l/kg.

A single dose of i.m. depot TAA has far longer lasting (up to 4 weeks) effects on HPA axis activity than the similar i.v. dose of TAA-DHP (effect lasting <72 h). This was confirmed by reduced basal cortisol levels as well as by cortisol responses to

**Table 1 Pharmacokinetic parameters (means ± S.E.M., n = 10) for TAA after a single-dose (0·2 mg/kg) long-acting i.v. versus i.m. depot TAA administration**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TAA-DHP (i.v.)</th>
<th>TAA (i.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (nmol/l)</td>
<td>5·8 ± 0·2</td>
<td>3·1 ± 0·3</td>
</tr>
<tr>
<td>tmax (h)</td>
<td>0·6 ± 0·1</td>
<td>1·5 ± 0·1</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>13·9 ± 1·3</td>
<td>125·9 ± 15·8*</td>
</tr>
<tr>
<td>AUC0–N (nmol/h/l)</td>
<td>17·9 ± 1·7</td>
<td>134·2 ± 8·4*</td>
</tr>
<tr>
<td>MRT0–N (h)</td>
<td>10·7 ± 1·3</td>
<td>159·8 ± 21·2*</td>
</tr>
<tr>
<td>CL (l/ml/min/kg)</td>
<td>0·5 ± 0·1</td>
<td>0·1 ± 0·0</td>
</tr>
<tr>
<td>Vz (l/kg)</td>
<td>0·6 ± 0·1</td>
<td>0·6 ± 0·1</td>
</tr>
</tbody>
</table>

Cmax, maximum plasma concentration; tmax, time of Cmax; t1/2, terminal elimination half-life; AUC0–N, area under the plasma concentration–time curve; MRT0–N, mean residence time; CL, clearance; Vz, volume of distribution. *P < 0·001 vs intravenous TAA-DHP.
ACTH challenge. Indeed, the pharmacokinetic profiles of the two TAA formulations corroborate this finding.

The observed depot TAA-induced decrease in basal and ACTH-stimulated cortisol levels over a month can be explained by a reduced function of the adrenal cortex to synthesise and secrete cortisol. Upstream suppressed CRH and ACTH release by feedback through elevated cortisol is well known (Livanou et al. 1967, Gulliver & Eid 2005) and we predict that injections of TAA formulations may have similar feedback effects. Another explanation for the prolonged suppressive effect of the single-dosed depot TAA on HPA axis activity is possibly the systemic accumulation of the drug as the pharmacokinetic observations clearly point to this phenomenon. First, the MRT of the i.m. TAA was about 15-fold longer than that of i.v. TAA-DHP. Secondly, the half-life of TAA after i.m. administration (130 h) was longer than the i.v. (14 h) administration. TAA was detectable in plasma over 4 weeks after i.m. administration, suggesting that the less soluble depot formulation is absorbed for a longer period of time than the soluble i.v. TAA formulation, as suggested also for the intra-articular depot TAA administration (Kumar et al. 2004). Moreover, and thirdly, the AUC after a single i.m. depot TAA was 7-3-fold larger than after i.v. TAA-DHP administration, indicating the significant prolonged absorption rate of the i.m. depot TAA due to a ‘flip-flop’ pharmacokinetic scenario. This means that the ascending phase of the curve (0–2 h) represents the disposition of TAA, and the descending part the rate-limiting process of TAA release from the muscle into the circulation system (Fig. 2B). Therefore, because of this higher AUC value, one may predict that the suppressed response of the HPA axis is strongly associated with the prolonged effects of the depot formulation of TAA. Fourthly, the total body clearance was low in the depot form (Table 1), the total body clearance was low in the depot formulation of TAA-DHP in the present study were in agreement with the i.v. TAA pharmacokinetics in human studies (Möllmann et al. 1985, Derendorf et al. 1995, Rohtagi et al. 1995).

With regard to topical administration of TAA and its effect on HPA axis function, there are some discrepancies. For example, a repeated local intra-articular administration of TAA (40 mg) caused in paediatric patients signs of Cushing’s syndrome that appeared 4–6 weeks after TAA delivery and lasted 4–6 months after the last dose of TAA (Kumar et al. 2004). TAA was still detectable in the plasma and urine for 4–5 months after the last dose of the steroid, which was much longer than in our study, perhaps due to drug accumulation, but the distinct patterns of the pharmacodynamic and pharmacokinetic profiles support in part our findings obtained from the single-dose i.m. depot TAA injection. But in contrast to the intra-articular TAA adverse effects, the intraspinal TAA administration which is beneficial in patients with multiple sclerosis did not seem to affect the adrenocortical cortisol release (Neu et al. 1978, Hoffmann et al. 2006); indeed, the metabolic fate of the intraspinal TAA is presently not well known. In addition, it was shown that a repeated intranasal administration of high-dose aqueous depot TAA does not seem to be absorbed and is unlikely to cause clinically relevant HPA axis suppression in asthmatic patients (Sorkness et al. 1999, Skoner et al. 2003, Bachert et al. 2004).

In summary, our findings indicate that after a single equivalent dose of the i.m. depot TAA or the i.v. long-acting TAA-DHP, there was an excellent correlation between the pharmacokinetic and pharmacodynamic profiles. The HPA axis and the adrenocortical activity were more persistently impaired by the depot formulation than by the long-acting i.v. TAA-DHP, indicating a cumulative effect of TAA on the adrenal refractoriness over at least 1 month. In conclusion, the regular clinical use, for example, at weekly intervals, of depot GCs may necessarily worsen the conditions of adrenal crisis; thus, in patients who are in need of corticosteroid therapy careful re-estimation of the pharmacokinetics and pharmacodynamics of depot GC formulations should be considered.

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