A placebo-controlled study of three osteocalcin assays for assessment of prednisolone-induced suppression of bone turnover

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Abstract

Serum osteocalcin is a sensitive marker of suppressive effects of exogenous glucocorticoids on bone turnover. It has been suggested, however, that the degree of suppression detected by different assays may vary. Whether discrepancies between various assays influence conclusions from group studies of exogenous glucocorticoids has not been evaluated. The aim of the present study was to compare the CAP fluoroimmunoassay (FEIA), OSTK-PR and ELSA-OSTEO assays for assessment of prednisolone-induced effects on serum osteocalcin. Twelve men and eight premenopausal women aged 19–45 (mean 31) years were studied. All subjects were healthy. The design was a randomised double-blind, placebo-controlled parallel-group study with 2 days run-in, 3 days treatment and 4

days run-out. During run-in and run-out no medication was given. During the treatment period the subjects took either 20 mg prednisolone twice daily or placebo. Blood was collected on the last day of each period. Intra- and intergroup comparisons showed prednisolone treatment to be associated with a statistically significant suppression of osteocalcin which was detected by all assays (ANOVA; $P < 0.0001$). In the individual subjects the response to prednisolone was the same for each assay. The CAP FEIA, OSTK-PR and ELSA-OSTEO assays seem equally sensitive for evaluation of osteocalcin in group studies of oral glucocorticoids.

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Introduction

Osteocalcin is the most abundant noncollagenous protein of bone. It is synthesised only by the osteoblasts, and changes in osteoblast activity correlate with variations in serum concentrations of osteocalcin (Nielsen *et al.* 1988b). Serum osteocalcin has been established as a sensitive marker of suppressive effects of exogenous glucocorticoids on bone turnover (Nielsen *et al.* 1988a,b, Meeran *et al.* 1995). Systemic glucocorticoids cause a dose-dependent reduction in osteocalcin levels (Hodsman *et al.* 1991, Wolthers *et al.* 1993). It has been suggested, however, that the degree of glucocorticoid suppression detected by various assays may vary, whereby interpretation of osteocalcin in individual patients treated with exogenous glucocorticoids becomes difficult (Masters *et al.* 1994). Whether discrepant test results may influence conclusions from group studies of exogenous glucocorticoids has not been evaluated. Therefore, the aim of the present study was to assess whether prednisolone treatment is associated with any adverse effects on serum osteocalcin using three osteocalcin assays.

Subjects and Methods

Twelve men and eight women aged 19–45 (mean 31) years, doctors and nurses, laboratory workers and students

at our department, were asked and agreed to participate in the study. The subjects were carefully screened by medical examination and proved healthy without any history of metabolic or renal disease. None of them had ever received treatment with exogenous glucocorticoids and no other drugs were taken 2 months before or during the study period. All subjects were non-smokers. None of the females was pregnant or had menstruation during the study. All subjects entered the study on the same day, and they continued their usual activities throughout the study. The study was approved by the local ethics committee, and each subject gave informed consent before the study.

The design was a randomised double-blind, placebo-controlled parallel-group study with 2 days run-in, 3 days treatment and 4 days run-out. During run-in and run-out no medication was given. During the treatment period the subjects took either prednisolone (40 mg per day) or placebo. Allocation to treatment was performed by means of a computerised randomisation scheme prepared in balanced blocks. Prednisolone and placebo were divided into two daily doses and taken in the morning at 0700 h and in the evening at 1900 h. The tablets, identical in size and appearance, were delivered in identical glasses labelled with case number and prescription. Tablets were counted before and after the treatment period.

Table 1 Characteristics of the osteocalcin assays

Assay	Type	Antibody	Variation (%)	
			Intra-assay	Interassay
CAP	FEIA	Antihuman OC, mAb	6.2	4.1
ELSA-OSTEO	Sandwich RIA	Antihuman OC, mAb	5.4	5.0
OSTK-PR	Competitive RIA	Rabbit anti-bovine OC	5.5	5.2

OC, osteocalcin; mAb, monoclonal antibody.

Glass tubes without clot accelerators were used for the collection of blood which took place during the afternoon between 1300 and 1700 h on the last day of the run-in, treatment and run-out periods. The blood samples were taken at roughly the same time (that is, within 30 min) and were centrifuged at 4000 r.p.m. for 10 min within 1 h of collection. After centrifugation serum samples were stored in plastic tubes at -80°C , and they were thawed only once, i.e. immediately before they were batch assayed at completion of the study, each sample being run in all three assays at the same time. Haemolysed samples were not used for analysis. The osteocalcin assays used were Pharmacia Osteocalcin CAP fluoroimmunoassay (FEIA) (Kabi Pharmacia Diagnostics AB 1994) and CIS Human Osteocalcin IRMA (ELSA-OSTEO) (Cis Bio International 1994) and RIA (OSTK-PR) (CIS Bio International 1995). Details of the assays are given in Table 1. In the CAP FEIA osteocalcin standards were calibrated against a reference standard, which was prepared according to a modified method (Taylor *et al.* 1988) and quantified by amino acid analysis. In the ELSA-OSTEO and OSTK-PR assays animal proteins (ELSA-OSTEO) and bovine osteocalcin and human proteins (OSTK-PR) were used in lyophilised standards. Normal ranges of serum osteocalcin depend on sex, age and the assay used (Gundberg *et al.* 1985, Tarallo *et al.* 1990). Preliminary assessments using the CAP FEIA, OSTK-PR and ELSA-OSTEO assays have found normal ranges of 1–25, 1–18 and 5–35 $\mu\text{g/l}$ respectively, in 20- to 50-year-old men, and of 1–15, 1–13 and 8–36 $\mu\text{g/l}$ respectively, in 20- to 50-year-old, premenopausal women (Kabi Pharmacia Diagnostics AB 1994, CIS Bio International 1994, 1995).

Statistics

Data are described as means $\pm$ standard error of the means. To evaluate differences between run-in, treatment and run-out periods in the placebo group, one-way analysis of variance on ranks was performed. Differences within the prednisolone group were compared by one-way analysis of variance followed by Student–Newman–Keuls multiple range test. Intergroup comparisons were performed with Students’s *t*-test for unpaired samples if data fulfilled conditions for parametric analysis, otherwise the Mann–

Whitney rank sum test was used. To assess whether the response of osteocalcin to prednisolone was the same for each assay the percentage change of prednisolone to run-in values was evaluated by the method described by Bland & Altman (1986). The 5% level of significance was used. All statistics were performed using the SOLO Statistical Software package (BMDP Statistical Software, Los Angeles, CA, USA).

Results

Six males and three females (age range (mean): 27–44 (33) years) were allocated to treatment with prednisolone, and six males and five females (19–45 (29) years) were allocated to placebo treatment. Height, weight and body surface area (range (mean)) were 161–184 (174) cm, 60–81 (69) kg, 1.7–2.0 (1.8) m^2 in the prednisolone group, and 165–200 (177) cm, 57–114 (72) kg, 1.6–2.5 (1.9) m^2 in the placebo group. There were no significant demographic differences between the groups. Compliance with dosage regimen was 100% in both groups during all periods. The treatment blood sample from one male in the placebo group was lost, and a male in the prednisolone group forgot his run-out appointment and, therefore, did not have blood collected during that period.

Individual values of serum concentrations of osteocalcin during run-in, treatment and run-out are shown in Fig. 1. All data showed a Gaussian distribution. Group means and the results of intra- and intergroup comparisons are given in Table 2. The intra-group comparisons show that prednisolone treatment was associated with a statistically significant suppression of osteocalcin which was detected by all assays. Furthermore, osteocalcin values returned to pretreatment levels during run-out. In the placebo group osteocalcin levels were constant throughout all periods. The results of the intergroup analyses based on each assay were consistent with the intra-group comparisons. Figure 2 shows the plots of the pairwise comparisons of the CAP FEIA, OSTK-PR and ELSA-OSTEO assays respectively, in the prednisolone-treated group based on the percentage of prednisolone to run-in values in all subjects. In the individual subjects the response to prednisolone was similar for each assay.

Figure 1 Run-in, treatment and run-out serum concentrations of osteocalcin measured with the CAP FEIA, OSTK-PR and ELSA-OSTEO assays in 11 subjects treated with placebo (upper panel) and 9 subjects treated with prednisolone 20 mg twice daily (lower panel).

Discussion

Most of the commercially available osteocalcin assays are based on radioimmunoassay techniques some of which use polyclonal, others monoclonal antibodies (Delmas *et al.* 1990, Diaz Diego *et al.* 1994, Garnero *et al.* 1994, Masters *et al.* 1994, Blumsohn *et al.* 1995, Cecchetti *et al.* 1995). The OSTK-PR assay applies polyclonal antibodies raised against bovine osteocalcin. It is the most widely used reference. We, therefore, compared this and an assay which applies monoclonal antibodies raised against human osteocalcin, the ELSA-OSTEO assay, with the recently introduced fluorescence immunoassay based on immunoCAP technology using monoclonal antibodies raised against human osteocalcin.

The absolute osteocalcin values determined by different assays may depend on assay type (RIA, FEIA), standardisation (human, bovine), antibody type (monoclonal, polyclonal), calcium dependency (Tracy *et al.* 1990), sensitivity, recovery, precision and specificity of the assay (human, bovine, intact osteocalcin molecule, C-terminal end of the molecule) (Delmas *et al.* 1990). Different assays may measure different fragments of the osteocalcin polypeptide (Deftos *et al.* 1992). In the ELSA-OSTEO assay the antibody tracer is directed against part of the

osteocalcin molecule whereby intact osteocalcin as well as degradation products may be measured (Diaz Diego *et al.* 1994). A combination of one or more of the above-mentioned factors may explain why higher values were detected by the ELSA-OSTEO assays compared with the CAP FEIA which detects intact osteocalcin only (Kabi Pharmacia Diagnostics AB 1994). The epitopes bound by the antiserum used in the OSTK-PR assay have not been described and further studies addressing specificity and reactivity of osteocalcin assays are needed before these interassay variations can be fully understood. The results of the present study, however, confirm the reliability of the CAP FEIA for determination of serum osteocalcin in glucocorticoid-treated subjects. During prednisolone treatment reduced osteocalcin levels were detected by all assays in each patient and overall. So, although the degree of suppression may vary between assays and may confound osteocalcin data in patients with various bone disorders (Masters *et al.* 1994), the CAP FEIA, OSTK-PR and ELSA-OSTEO assays seem equally reliable for evaluation of osteocalcin in group studies of exogenous glucocorticoids.

Besides dose, the degree of suppression of serum concentrations of osteocalcin observed in individuals during glucocorticoid treatment may depend on several factors

Table 2 Serum concentrations (g/l) of osteocalcin (group means ± S.E.M.) based on the CAP FEIA, OSTK-PR and ELSA-OSTEO assays and intra- and intergroup comparisons (*P* values) in 11 subjects treated with placebo and 9 subjects treated with prednisolone (20 mg twice daily).

	CAP FEIA	OSTK-PR	ELSA-OSTEO
Placebo group			
Run-in (<i>n</i> =11)	7.55 ± 1.17	9.45 ± 1.07	28.64 ± 3.92
Treatment (<i>n</i> =10)	7.41 ± 1.49	9.53 ± 1.31	29.42 ± 4.82
Run-out (<i>n</i> =11)	8.57 ± 1.40	10.21 ± 1.14	29.34 ± 4.18
Statistical comparisons			
ANOVA	0.54*	0.76*	0.95*
Prednisolone group			
Run-in (<i>n</i> =9)	6.10 ± 0.64	8.63 ± 0.83	22.41 ± 1.89
Treatment (<i>n</i> =9)	2.00 ± 0.31	2.96 ± 0.43	7.71 ± 1.10
Run-out (<i>n</i> =8)	6.33 ± 0.87	8.19 ± 1.08	20.64 ± 2.55
Statistical comparisons			
ANOVA	<0.0001	<0.0001	<0.0001
Run-in vs treatment	<0.05	<0.05	<0.05
Treatment vs run-out	<0.05	<0.05	<0.05
Run-in vs run-out	NS	NS	NS
Placebo vs prednisolone group			
Statistical comparisons			
Run-in	0.32	0.57	0.32**
Treatment	<0.0001**	<0.0005	<0.001**
Run-out	0.23	0.23	0.12

*ANOVA on ranks; **Mann–Whitney rank sum test.
NS, not significant.

such as individual variations in metabolism and sensitivity to exogenous glucocorticoids, concurrent administration of topical glucocorticoids, potency of the drug used and administration regimen (Reid 1989, Wolthers *et al.* 1993, Knutsson *et al.* 1995, Morice *et al.* 1996). Long term treatment is associated with a significant risk of osteopenia (Reid 1989). To reduce the risk in many conditions short term treatment regimens are used (Toogood & Hodsman 1991, Lems *et al.* 1993, Wolthers *et al.* 1996). Since an

exponential decline in bone mass has been found with the highest bone loss within 6 months of treatment and a much slower rate of loss after 12 months of treatment (LoCascio *et al.* 1990), short term evaluations of bone turnover focusing specifically on the relation between duration of treatment and duration of possible adverse effects on bone turnover are needed. In the present study we wanted to assess an administration regimen which is used in a variety of conditions (Toogood & Hodsman

Figure 2 Means (x axis) and differences (y axis), expressed as percentages of run-in levels, of pairwise comparisons of osteocalcin values assessed by the CAP FEIA, OSTK-PR and ELSA-OSTEO assays during prednisolone treatment. The different marks identify the 9 individual subjects in each panel.

1991). Our findings are in accord with reports that prednisone and prednisolone 20 mg twice daily for 5 and 10 days respectively, are associated with suppressed osteocalcin levels (Nielsen *et al.* 1988b, Hodsmann *et al.* 1991, Lane *et al.* 1996). The duration of the suppression of osteocalcin after a single administration of 10 mg prednisone seems to be approximately 16 h (Nielsen *et al.* 1988a). The duration of suppression after withdrawal of several days of treatment, however, has not previously been assessed. This is an important factor to consider since a cumulative suppressive effect may play a key role in the early and rapid bone loss which occurs during treatment with exogenous corticosteroids (Reid 1989, LoCascio *et al.* 1990, Worth *et al.* 1994). The present findings have provided evidence that 4 days after withdrawal of a 3-day course of prednisolone (20 mg twice daily) bone turnover is unaffected. So, the risk of long term effects of short term treatment can be ignored. The risk of a suppressive effect on bone turnover from intermittent short term treatment with exogenous glucocorticoids, however, may need further evaluation in the intermediate and long term perspectives.

References

- Bland JM & Altman DG 1986 Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986 **i** 307–310.
- Blumsohn A, Hannon RA & Eastell R 1995 Apparent instability of osteocalcin in serum as measured with different commercially available immunoassays. *Clinical Chemistry* **41** 318–319.
- Cecchetti M, Bellometti S, Torri G & Galzigna L 1995 Comparison of commercial osteocalcin assay kits in evaluating osteoporosis. *Current Therapeutic Research* **56** 163–168.
- Cis Bio International 1994. ELSA-OSTEO. Gif-Sur-Yvette Cedex, France.
- Cis Bio International 1995. OSTK-PR. Gif-Sur-Yvette Cedex, France.
- Defos LJ, Wolfert RL, Hill CS & Burton DW 1992 Two-site assays of bone gla protein (osteocalcin) demonstrate immunochemical heterogeneity of the intact molecule. *Clinical Chemistry* **38** 2318–2321.
- Delmas PD, Christiansen C, Mann KG & Price PA 1990 Bone Gla protein (osteocalcin) assay standardization report. *Journal of Bone and Mineral Research* **5** 5–11.
- Diaz Diego EM, Guerrero R & de la Piedra C 1994 Six osteocalcin assays compared. *Clinical Chemistry* **40** 2071–2077.
- Garnero P, Grimaux M, Seguin P & Delmas PD 1994 Characterization of immunoreactive forms of human osteocalcin generated *in vivo* and *in vitro*. *Journal of Bone and Mineral Research* **9** 255–264.
- Gundberg CM, Markowitz ME, Mizruchi M & Rosen JF 1985 Osteocalcin in human serum: a circadian rhythm. *Journal of Clinical Endocrinology and Metabolism* **60** 736–739.
- Hodsmann AB, Toogood JH, Jennings B, Fraher LJ & Baskerville JC 1991 Differential effects of inhaled budesonide and oral prednisolone on serum osteocalcin. *Journal of Clinical Endocrinology and Metabolism* **72** 530–540.
- Kabi Pharmacia Diagnostics AB 1994 Pharmacia CAP system osteocalcin FEIA. Uppsala, Sweden: Kabi Pharmacia Diagnostics AB.
- Knutsson U, Stierna P, Marcus C, Carlstedt Duke J, Carlstrom K & Bronnegard M 1995 Effects of intranasal glucocorticoids on endogenous glucocorticoid peripheral and central function. *Journal of Endocrinology* **144** 301–310.
- Lane SJ, Vaja S, Swaminathan R & Lee TH 1996 Effects of prednisolone on bone turnover in patients with corticosteroid resistant asthma. *Clinical and Experimental Allergy* **26** 1197–1201.
- Lems WF, Jacobs JW, van den Brink HR, van Rijn HJ & Bijlsma JW 1993 Transient decrease in osteocalcin and markers of type 1 collagen turnover during high-dose corticosteroid pulse therapy in rheumatoid arthritis. *British Journal of Rheumatology* **32** 787–789.
- LoCascio V, Bonucci E, Imbimbo B, Ballanti P, Adami S, Milani S, Tartarotti D & DellaRocca C 1990 Bone loss in response to long-term glucocorticoid therapy. *Bone and Mineral* **8** 39–51.
- Masters PW, Jones RG, Purves DA, Cooper EH & Cooney JM 1994 Commercial assays for serum osteocalcin give clinically discordant results. *Clinical Chemistry* **40** 358–363.
- Meeran K, Hattersley A, Burrin J, Shiner R & Ibbertson K 1995 Oral and inhaled corticosteroids reduce bone formation as shown by plasma osteocalcin levels. *American Journal of Respiratory and Critical Care Medicine* **151** 333–336.
- Morice AH, Morris D & Lawson MP 1996 A comparison of nebulized budesonide with oral prednisolone in the treatment of exacerbations of obstructive pulmonary disease. *Clinical Pharmacology and Therapeutics* **60** 675–678.
- Nielsen HK, Charles P & Mosekilde L 1988a The effect of single oral doses of prednisone on the circadian rhythm of serum osteocalcin in normal subjects. *Journal of Clinical Endocrinology and Metabolism* **67** 1025–1030.
- Nielsen HK, Thomsen K, Eriksen EF, Charles P, Storm T & Mosekilde L 1988b The effects of high-dose glucocorticoid administration on serum bone gamma carboxyglutamic acid-containing protein, serum alkaline phosphatase and vitamin D metabolites in normal subjects. *Bone and Mineral* **4** 105–113.
- Reid IR 1989 Pathogenesis and treatment of steroid osteoporosis. *Clinical Endocrinology* **30** 83–103.
- Tarallo P, Henny J, Fournier B & Siest G 1990 Plasma osteocalcin: biological variations and reference limits. *Scandinavian Journal of Clinical and Laboratory Investigation* **50** 649–655.
- Taylor AK, Linkhart SG, Mohan S & Baylink DJ 1988 Development of a new radioimmunoassay for human osteocalcin: evidence of a midmolecule epitope. *Metabolism* **37** 872–877.
- Toogood JH & Hodsmann AB 1991 Effects of inhaled and oral corticosteroids on bone. *Annals of Allergy* **67** 87–90.
- Tracy RP, Andrianorivo A, Riggs BL & Mann KG 1990 Comparison of monoclonal and polyclonal antibody-based immunoassays for osteocalcin: a study of sources of variation in assay results. *Journal of Bone and Mineral Research* **5** 451–461.
- Wolthers OD, Riis BJ & Pedersen S 1993 Bone turnover in asthmatic children treated with oral prednisolone or inhaled budesonide. *Pediatric Pulmonology* **16** 341–346.
- Wolthers OD, Heuck C, Ternowitz T, Heickendorf L, Nielsen HK & Frystyk J 1996 The insulin-like growth factor axis and bone and collagen turnover in children with atopic dermatitis treated with topical glucocorticosteroids. *Dermatology* **192** 337–342.
- Worth H, Stammen D & Keck E 1994 Therapy of steroid-induced bone loss in adult asthmatics with calcium, vitamin D, and a diphosphonate. *American Journal of Respiratory and Critical Care Medicine* **150** 394–397.

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