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Note

Determination of methylprednisolone and methylprednisolone acetate in synovial fluid using high-performance liquid chromatography

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Methylprednisolone acetate (MPA) administered intraarticularly is widely used in both human and veterinary medecine for the relief of various acute and chronic joint diseases [1]. After its administration, MPA must be hydrolysed

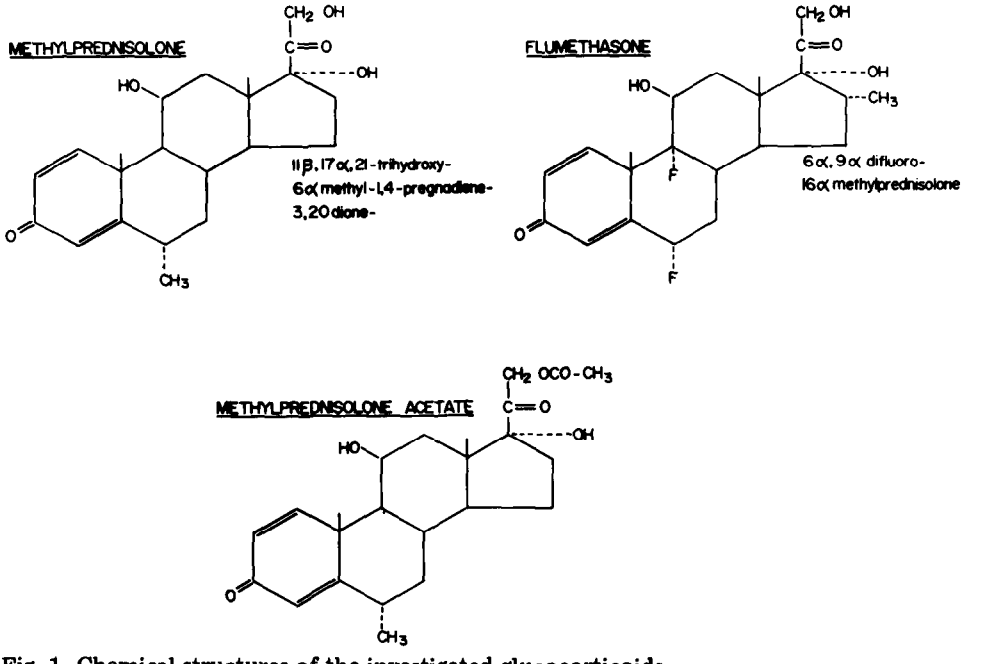

Fig. 1. Chemical structures of the investigated glucocorticoids.

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to the pharmacologically active parent drug, methylprednisolone (MP) (Fig. 1). In whole blood, a rapid rate constant for MPA hydrolysis has been reported [2]. In contrast, no information is available concerning the hydrolysis of MPA to MP in synovial fluid.

Several mehods [3, 4] have been described to measure selectively the concentration of MP in plasma by use of high-performance liquid chromatography (HPLC); however, no method has been developed for the simultaneous assay of MP and MPA in various biological fluids.

Using the advantages of a common normal-phase chromatographic method that has been successfully applied to a large number of steroids [5, 6], the present report describes a sensitive and specific procedure for the simultaneous determination of MP and MPA in synovial fluid.

EXPERIMENTAL

Apparatus

A constant-volume high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), consisting of a Model M45 pump, a U6K injector and a Model M440 detector, was used. The column (10 × 0.8 cm I.D.) was packed with 10-μm silica gel (Radial Pak B, Waters Assoc.) and was included in a radial compression system (Module RCM 100, Waters Assoc.).

Reagents

MP, MPA and flumethasone reference standard were purchased from Sigma (St. Louis, MO, U.S.A.). Dichloromethane was obtained from Farmitalia Carlo-Erba (Milan, Italy).

Standards

Each steroid was dissolved in methanol at a concentration of 1 mg/ml. The working standard mixture was prepared by diluting the stock solution 1:100 in the elution solvent. Flumethasone was used as internal standard at a concentration of 1 mg/ml in methanol; a working solution was prepared by diluting the stock solution 1:10 in methanol.

Operating conditions

The mobile phase was prepared by mixing exact volumes of dichloromethane, methanol and glacial acetic acid (96.8:2.4:0.8, v/v). The solution was stirred and degassed. A constant flow-rate of 1.4 ml/min was maintained. The radial compression pressure was 100 bars and inlet pressure was 15 bars.

The ultraviolet detector was set at 254 nm; the sensitivity of the detector was 0.001 a.u.f.s. (absorbance units full scale). The system was operated at ambient temperature (18—20° C).

Retention time and selectivity

Normal-phase liquid chromatography showed a high selectivity in the separation of glucocorticoids; a good separation was obtained in 8 min (Table I).

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF SELECTED GLUCOCORTICOIDS

Component	Capacity factor (k')	Retention time (min)
Methylprednisolone acetate	1.2	4
Flumethasone	2.3	6
Methylprednisolone	3.4	8

Extraction procedure

In a 30-ml tube (Corex, U.S.A.) were placed 100 μl of sample, 10 μl of the working solution of internal standard (equivalent to 0.1 μg of flumethasone), 1 ml of 0.1 *M* sodium hydroxide and 10 ml of dichloromethane. The tubes were shaken for 10 min and centrifuged at 8400 *g* for 10 min. The dichloromethane layer was aspirated off and evaporated at 40°C under a stream of nitrogen gas to prevent oxidation. The sample extract was reconstituted with 100 μl of eluent, mixed using a vortex mixer, and the total was then injected on to the column.

Calibration

Pooled bovine synovial fluids were spiked with MP and MPA to give concentrations in the range 0.02—1 μg/ml. A constant amount (10 μl) of working solution of the internal standard was added to each sample. Pooled synovial fluid samples were run through the procedure and calibrations curves constructed by plotting the peak height ratio for each compound with respect to the internal standard against the amount of compound added to each synovial sample. Least-squares regression analysis was used to determine the slope, intercept and correlation coefficient for each compound in the concentration range tested (Table II). The response of the HPLC system was linear from 0.02 to 1 μg/ml for each of the two compounds.

The analytical recovery of the steroids was measued by comparing the chromatographic peak heights from the analysis of biological samples which were spiked with 100 ng of MP and MPA to the peak height resulting from a direct injection. The recovery of all steroids from synovial fluid was 80—85% when approximately 85—90% of the dichloromethane layer was available for evaporation.

TABLE II

LEAST-SQUARES REGRESSION STATISTICS FOR HPLC CALIBRATION DATA OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE ACETATE IN SPIKED SAMPLES

Component	Slope	Intercept	Correlation coefficient (n = 6)
Methylprednisolone	0.0105	+0.045	0.997
Methylprednisolone acetate	0.0405	+0.015	0.998

Precision

The intraassay variation was determined by analysing synovial fluid samples spiked with 100 ng of MP and MPA. Coefficients of variation were 5.8% (n = 8) and 4.9% (n = 8), respectively.

The interday variability of the assay over a period of a week (n = 5) was determined by analysing samples spiked with 100 ng of MP and MPA. Coefficients of variation were 8.7% and 7.5%, respectively.

Drug disposition study

To test ability of this method to detect MP and MPA in synovial fluid of treated animals, 200 mg of methylprednisolone acetate (Depomedrol; Upjohn, Paris, France) were injected into the tibiotarsal joint of a cow. Synovial fluid samples were collected every day during the first week, and every three days for two months after administration. In order to prevent hydrolysis of the parent compound, samples were immediately stored at −21°C until analysis.

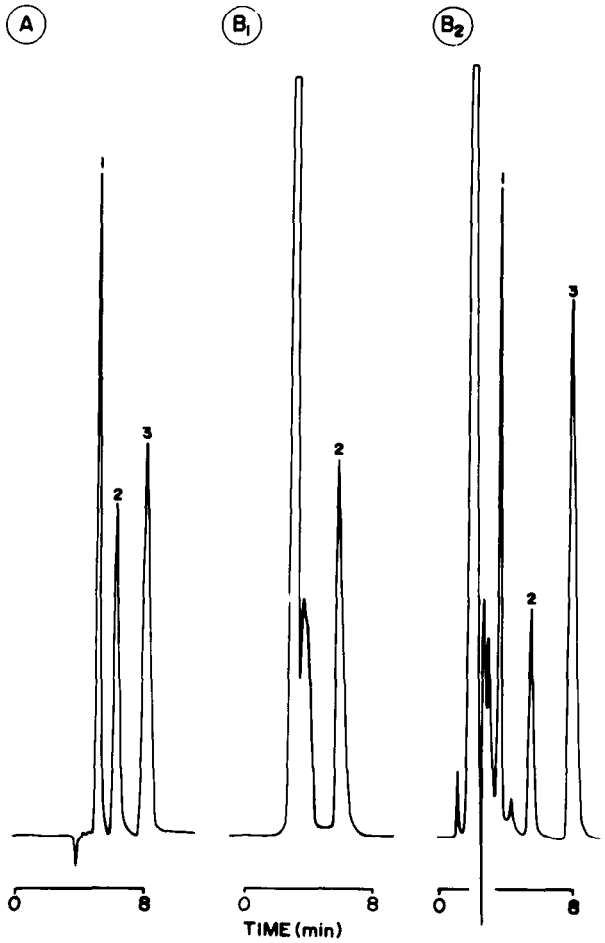

Fig. 2. (A) Chromatogram of a mixture of 100 ng each of MPA, flumethasone and MP. (B_1) Chromatogram of extracted blank synovial fluid containing only internal standard. (B_2) Chromatogram of extracted synovial fluid after administration of MPA with serum levels of 230 ng/ml for MP and 100 ng/ml for MPA. Peaks: 1 = methylprednisolone acetate, 2 = flumethasone, 3 = methylprednisolone.

RESULTS AND DISCUSSION

The purpose of this study was to develop a precise and sensitive technique which would allow the simultaneous determination of MP and its ester (MPA) in synovial fluid. In addition, a suitable technique for this purpose must include the choice of a suitable internal standard with good separation from the glucocorticoids to be measured. In this respect flumethasone was used and the method described in this report meets these criteria.

Fig. 2A shows a chromatogram of a mixture of 100 ng each of MPA, flumethasone and MP, demonstrating the clear separation of the glucocorticoids using the column and the mobile phase described above. The longest retention was approximately 8 min for MP, which is the least polar of the three assayed compounds.

Fig. $2B_1$ shows a typical chromatogram of a synovial fluid sample, spiked with internal standard, from an untreated cow. No major endogenous peak that would interfere with the resolution of the glucocorticoids was encountered.

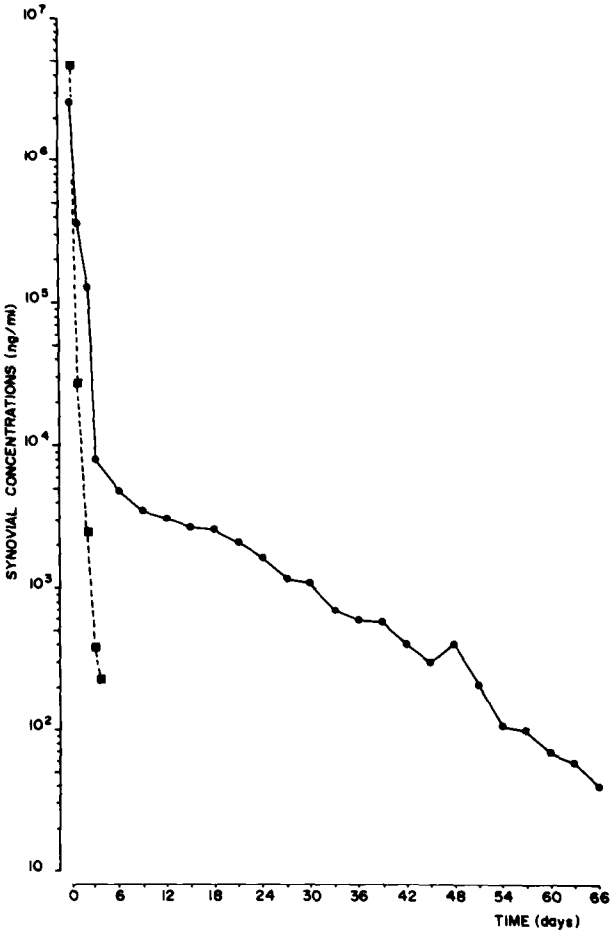

Fig. 3. Synovial concentration—time curve in the cow given a 200-mg intraarticular dose of MPA. ■, MPA; ●, MP.

Fig. $2B_2$ displays a chromatogram of a synovial fluid sample from an animal injected intraarticularly with MPA.

Fig. 3 presents a typical synovial fluid concentration—time profile of MP and MPA after intraarticular administration. MPA concentration declines very rapidly until 24 h and then more slowly. For MP the highest concentration was observed at the first sampling; thereafter, MP concentrations decreased very rapidly for the first week and more slowly between one and nine weeks after administration.

The fact that MP remains for so long in synovial fluid must be considered significant from a pharmacological point of view; other more extensive studies about the disposition of MPA in synovial fluid after intraarticular administration will be reported elsewhere.

In conclusion, a selective and sensitive method has been developed for MP and MPA assay in synovial fluid. The detection limit of the proposed method using the equipment described was 10 ng for MPA and 20 ng for MP based on a 100-μl synovial fluid sample; this could be enhanced by increasing the amount of sample, thus permitting the accurate measurement of concentrations as low as 50 ng/ml.

ACKNOWLEDGEMENTS

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