Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 2, pp. 177–181, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00

Short Communication

Simultaneous determination of the two main metabolites of deflazacort in human plasma by high-performance liquid chromatography

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Keywords: *Deflazacort; oxazolino steroid; normal-phase liquid chromatography; determination in human plasma.*

Introduction

Deflazacort†, Flantadin®, is a synthetic oxazolino steroid with a high glucocorticoid activity, which at present is in the launch phase as a systemic anti-inflammatory agent [1, 2]. The anti-inflammatory potency of deflazacort in comparison with prednisolone has been found to be 0.82 in rheumatoid arthritis and other diseases [3]. In clinical trials deflazacort has been observed to influence calcium balance and carbohydrate metabolism to lesser degrees than prednisolone [4, 5].

The metabolism of deflazacort in the rat, dog, monkey and man follows a common route consisting of rapid de-acetylation to deflazacort-21-alcohol (M II), and of 6-β-hydroxylation of this to form 6-β-hydroxy-deflazacort-21-alcohol (M III) (Fig. 1). The

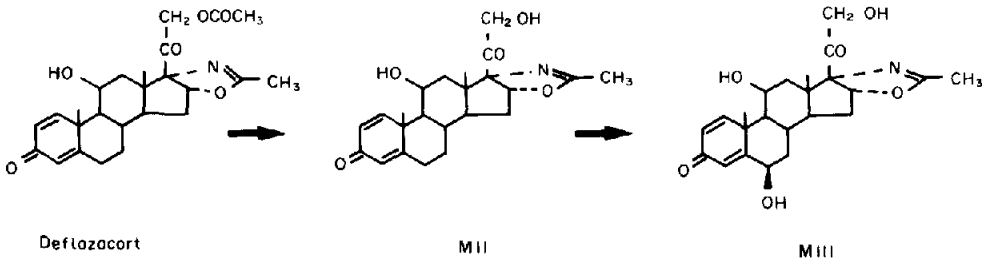

Figure 1
Major metabolic pathway of deflazacort.

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†International nonproprietary name, (11β,16)-21-(acetyloxy)-11-hydroxy-2'-methyl-5'H-pregna-1,4-dieno [16,17-d]oxazolo-3,29-dione. Flantadin® is the trade name of Gruppo Lepetit S.p.A.

parent drug is not detectable as the unchanged molecule. Both metabolites are present in plasma and urine: M II is the main compound in plasma, whilst M III predominates in urine [6, 7].

The aim of this work was to set up a sensitive and reproducible method for the simultaneous determination of M II and M III in human plasma for pharmacokinetic studies, taking into account the previous literature on glucocorticoid analysis in biological fluids [8, 9].

Materials and Methods

Chemicals

Deflazacort and M II were Lepetit working standards of appropriate high purity. M III was isolated and purified from human urine [7]. Prednisolone, used as the internal standard, was obtained from Sigma (St. Louis, MO, USA). Solvents and reagents were high purity grade. Distilled water was filtered through a Milli-Q-system, Millipore (Bedford, MA, USA). Plasma was obtained from Sprague Dawley rats and healthy human volunteers.

Apparatus

The HPLC system comprised a high pressure pump (Beckman Model 110), a valve injector (Beckman Model 340 Organizer), a fixed wavelength (254 nm) detector (Beckman Model 160) and a recorder (Beckman). A Zorbax Sil column (Dupont Instruments, Wilmington, DE, USA), 250 × 4.6 mm i.d., 5–6 μm, was used. Bransonic 12 ultrasonic equipment was supplied by Smithkline (Soest, The Netherlands). The shaker was a Continental Alter 2864 Model (Passoni, Milano, Italy).

Standard solutions

Internal standard. 10 mg of prednisolone was dissolved in 50 ml of acetonitrile–2-propanol (50:50; v/v) solution.

M II and M III. 10 mg of each compound was dissolved in 50 ml of the same solvent mixture.

Sample preparation

A 0.5 or 1 ml volume of human plasma was pipetted into a screw-cap tube containing 200 mg NaCl. The internal standard (500 ng) was added and after vortex mixing the sample was extracted twice with methylene chloride (10 and 5 ml) by shaking for 10 min at 300 inversions per min. After centrifuging at 3000 **g** for 10 min (ambient temperature) the aqueous layer and creamy interface were aspirated and discarded. The organic phase was then washed twice, by shaking as before, using 1 ml of an aqueous solution of 0.1 M sodium hydroxide saturated with sodium chloride and subsequently using 1 ml of a saturated aqueous solution of sodium chloride. After centrifuging, the aqueous phase was aspirated and discarded; 1 g of anhydrous sodium sulphate was added to dry the organic phase; after shaking and centrifuging, the organic phase was transferred to a conical tube and dried down under a stream of nitrogen at 37°.

Chromatography

The residue was redissolved in 50–100 μl of mobile phase and 20 μl was injected onto the HPLC column. Isocratic elution was made at ambient temperature using methylene

chloride–methanol (94:6; v/v), as the mobile phase; flow rate 1 ml min^{-1}. Measurements were taken by UV detection at 254 nm, considering the peak heights.

Results and Discussion

The described method affords a selective determination of metabolites II and III in human plasma as illustrated by Figs 2 and 3, with good chromatographic separation of the peaks. No unchanged deflazacort was detectable in plasma, thus it does not interfere in the determination of M II and M III.

In order to test precision, accuracy and linearity, plasma samples of untreated subjects were spiked with M II, M III and prednisolone by adding known amounts of the standard solutions. Samples were prepared for the 10–600 ng ml^{-1} concentration range of M II

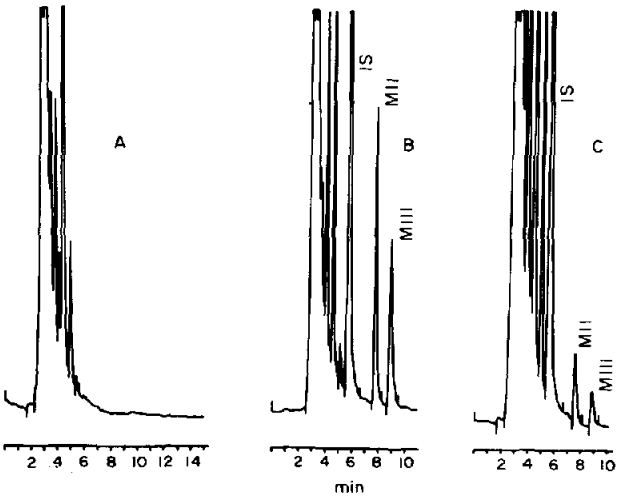

Figure 2
Chromatograms of control human plasma samples: (A) no addition; (B) containing 1 μg ml^{-1} each of internal standard (prednisolone), M II and M III; (C) containing 500 ng ml^{-1} of internal standard and 50 ng ml^{-1} each of M II and M III.

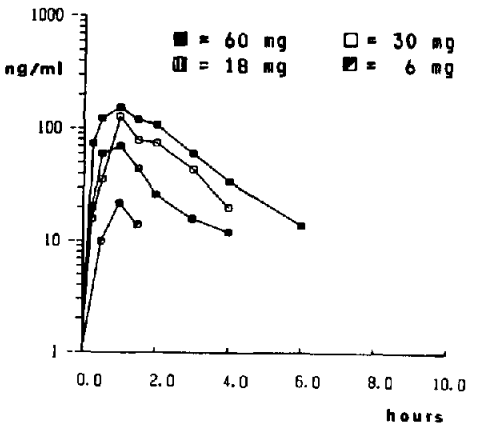

Figure 3
Semilogarithmic plot of M II plasma concentrations observed in one subject after oral administration of a 60, 30, 18 and 6 mg dose of deflazacort.

and M III, on the basis of the amounts expected to be in the plasma during kinetic studies. The repeatability of the assay was determined by analysing ten human plasma samples containing 80 and 200 ng ml^{-1} of both M II and M III, respectively, on two successive days. RSD values at 80 ng ml^{-1} were 4.2% (M II) and 6.6% (M III); at 200 ng ml^{-1}: 1.8% (M II) and 1.5% (M III) as summarized in Table 1.

The average recoveries of M II and M III over the range tested were 91.0–103.0% and 83.0–94.0%. These values show that the recovery of M III is lower than that of M II, presumably due to its higher polarity.

The accuracy was computed over 100 points of the human plasma calibration curve, with the following results: M II: 96.35% (RSD, 3.90%); M III: 88.31% (RSD, 4.55%).

The limit of detection (3 times the random noise level) is about 5 ng ml^{-1} for both metabolites.

The linear regression analysis provided the equations:

$$\text{M II}, \; y = 0.00205x - 0.00422, \; r = 0.9991,$$
$$\text{M III}, \; y = 0.00207x - 0.00425, \; r = 0.9990,$$

Table 1
Precision of the assay of deflazacort metabolites in human plasma

Concentration (ng ml^{-1})	Number of samples	M II		M III	
		mean %	RSD %	mean %	RSD %
10	6	91.0	3.5	83.0	4.4
20	6	91.4	7.6	83.6	7.8
40	6	93.2	6.3	85.8	2.4
60	8	96.6	6.2	84.2	5.7
80	5–2*	99.7	4.2	83.9	6.6
100	10	96.0	0.6	88.1	1.2
120	10	94.2	2.9	90.1	3.9
140	10	94.6	2.6	89.2	2.9
160	8	93.7	2.7	92.3	2.7
180	8	98.9	2.3	87.4	2.7
200	5–2*	103.0	1.8	93.2	1.5
400	8	99.1	1.7	94.0	4.8
600	6	101.2	1.7	93.2	6.2

*Precision repeatability test: the 10 samples were analysed on two successive days.
RSD = Relative Standard Deviation.

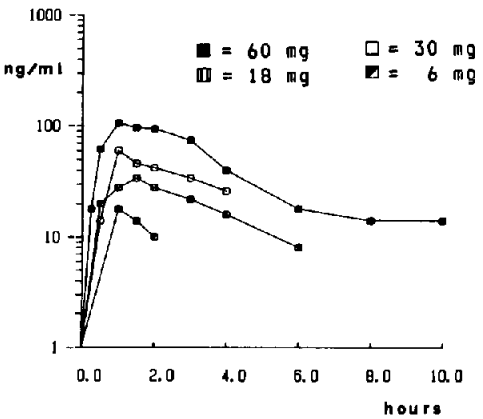

Figure 4
Semilogarithmic plot of M III plasma concentrations observed in one subject after oral administration of a 60, 30, 18 and 6 mg dose of deflazacort.

where y represents the amount of M II or M III found, x the amount added and r the correlation coefficient. The true values of M II and M III in a plasma sample can be found by these equations.

Pharmacokinetic studies

The reported assay method was applied in man to assess the plasma time course of M II and M III in twelve healthy volunteers after oral administration of 60, 30, 18 and 6 mg deflazacort (G. Poggi *et al.*, unpublished data). Figures 3 and 4 show the plasma concentrations of M II and M III observed in one subject after oral administration. Mean peak plasma concentrations of M II (23–91 ng ml^{-1}) and M III (15–62 ng ml^{-1}) were attained 1 h after administration. The concentrations of both compounds reached the detection limit 8–10 h after 60 mg administration and 2–3 h after 6 mg administration.

The method has proved to be reliable and suitable for routine analysis.

Acknowledgement: The authors wish to thank A. Perazzi for preparing metabolite III of deflazacort.

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[*Received for review 5 December 1985; revised manuscript received 31 July 1986*]