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Note

Determination of mofebutazone and its 4-hydroxy metabolite in plasma and urine by high-performance liquid chromatography

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Mofebutazone (MPB), the monophenyl analogue of phenylbutazone (DPB), is an anti-inflammatory drug which is said to be tolerated better than phenylbutazone. In man, its anti-inflammatory activity and toxicity are less than those of phenylbutazone[1,2]. In equine practice, however, it appears that mofebutazone is less effective than phenylbutazone. This can be attributed either to its limited solubility or to the low bioavailability when given orally. In order to study the pharmacokinetics of MPB in the horse, a method is needed which distinguishes between MPB and its metabolites or degradation products.

MPB can be rapidly oxidized to the 4-hydroxy compound, 1-phenyl-3,5-dioxo-4-hydroxy-4-*n*-butylpyrazolidine (4-OH-MPB), as shown in Fig. 1. The same product can also be formed during the analysis of MPB or even in pharmaceutical preparations, in which the percentage decomposition of MPB ranges between 5.2 and 10.2% and in some cases 76 and 82% of 4-OH-MPB is formed[3]. The method used for the determination of MPB and 4-OH-MPB in these pharmaceutical preparations[3] was based on thin-layer chromatography (TLC) followed by spectrophotometry, and is considered as unsuitable for the determination of both substances in biological fluids. Mofebutazone alone has been analysed qualitatively in screening methods by high-performance liquid chromatography (HPLC)[4,5]. In disposition studies in the rat[6,7] and man[8], mofebutazone was quantified using the compound [4-^{14}C]mofebutazone. However, due to the minor differences in TLC R_F values between MPB and 4-OH-MPB in these studies, the measured radioactivity could be related to the MPB concentration only if the metabolism was neglected. However, by taking appropriate measures to prevent oxidation, MPB and 4-OH-MPB can be simultaneously determined by HPLC using the method described here.

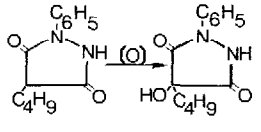

Fig. 1. Chemical structure of mofebutazone (MPB) and its oxidation product, 4-hydroxymofebutazone (4-OH-MPB).

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EXPERIMENTAL

Reagents and materials

Mofebutazone was obtained from Laboratoria Flandria (Zwijnaarde, Belgium) and its purity checked by HPLC. 4-OH-MPB was prepared by passing air through a solution of MPB in a mixture of acetone and toluene[9]. Its purity was checked by melting point (179°C) and HPLC analysis. The internal standard phenylbutazone was a gift from Byk Gulden (Konstanz, F.R.G.). All other chemicals were of reagent grades. Methanol was of HPLC grade obtained from Merck (Darmstadt, F.R.G.).

Instrumentation

The HPLC system was a Varian instrument with a variable wavelength UV detector set at 240 nm. Chromatography was done on a Nucleosil 5 C_{18} cartridge system (10 cm × 3 mm I.D.) from Chrompack (Antwerpen, Belgium). An appropriate disposable guard column (1 cm × 2.1 mm I.D.) was used.

Chromatographic conditions

The mobile phase for the analysis of MPB and 4-OH-MPB comprised 60% methanol and 40% water–acetic acid (30:1). The flow-rate was 0.4 ml/min.

Sample analysis

To 1 ml plasma in a screw-caped tube, 1 ml of 1 *M* acetate buffer (pH 5.3) and 0.1 ml of the internal standard solution [150 μg/ml phenylbutazone in ascorbic acid–methanol (1:100, w/v)] was added. The use of vitamin C was necessary to prevent oxidation of MPB during the extraction step. Extraction was performed by rolling on a commercial rolling apparatus with 5 ml diethyl ether for 5 min. After centrifugation (5 min), the organic phase was separated and evaporated under a nitrogen atmosphere at 40°C. The residue was redissolved in 0.1 ml methanol and 10 μl were injected into the liquid chromatograph.

Urine (0.5 ml) was analysed in a similar manner exept that 0.2 ml of internal standard were added. Centrifugation was not necessary.

Calibration curve

The linearity of the calibration was determined by adding 1, 2.5, 5, 8, 10, 15, 20 and 25 μg of MPB and 4-OH-MPB per ml to blank plasma samples. In a similar way, samples containing 5, 10, 20, 25, 50, 75 and 100 μg of MPB and 4-OH-MPB per ml urine were analysed. Four aliquots of each concentration were analyzed as described. The coefficients of variation were determined using replicate samples of plasma standards containing 5, 10 and 20 μg of MPB and 4-OH-MPB per ml, and of urine standards containing 10, 20 and 50 μg of MPB and 4-OH-MPB per ml.

Determination of extraction efficiency

The recoveries of the drugs were estimated from the changes in the peak-height ratios when the drugs were added to the plasma (urine) and the internal standard was added to the final extract compared to the peak height ratios when both the drugs and internal standard were added to the final extract.

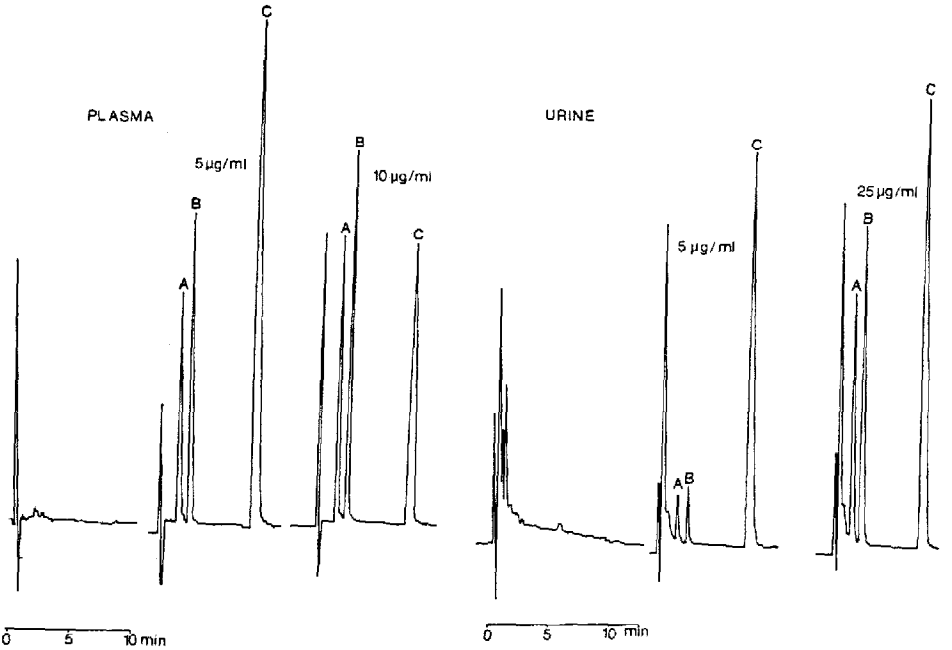

Fig. 2. Chromatograms obtained from residues of spiked plasma and urine. Peak: A = 4-OH-MPB; B = MPB; C = internal standard, DPB.

RESULTS AND DISCUSSION

Under the described chromatographic conditions, baseline resolution was achieved between MPB and 4-OH-MPB in plasma and urine extracts. Extracts from drug-free plasma and urine were found to be free from interfering peaks. Representative chromatograms from plasma and urine spiked with MPB and 4-OH-MPB are shown in Fig. 2. The retention times were 2.75, 3.60 and 8.50 min respectively for MPB, 4-OH-MPB and the internal standard DPB.

The peak-height ratio of MPB and 4-OH-MPB *versus* DPB was chosen as the quantitative measure of the detector response. Regression analysis of these data resulted in a correlation coefficient, r, greater than 0.998 for both MPB and 4-OH-MPB either in plasma or in urine.

The average recoveries (± S.D.) of MPB and 4-OH-MPB from plasma samples to which drug standard had been added at concentrations ranging from 5 to 20 µg/ml were determined to be 80.0 ± 3.7 and 67.7 ± 1.8% respectively (Table I). The extraction recovery from urine, however, was markedly higher giving values of 91.8 ± 3.5 and 95.2 ± 3.7% for MPB and 4-OH-MPB respectively. Compared to plasma, either a better separation between the organic phase and the urine or the lack of urinary protein binding could contribute to these higher recoveries. The data in Table I also suggest that there was practically no recovery dependence on concentration over the range of drug levels investigated.

The precision of the assay was assessed by analysis of plasma and urine samples containing known concentrations of MPB and 4-OH-MPB. The results of these de-

TABLE I

PERCENTAGE RECOVERY OF MPB AND 4-OH-MPB FROM PLASMA AND URINE

$n = 4$.

MPB and 4-OH-MPB concentration (μg/ml)	*Percentage recovery (means ± S.D.)*	
	MPB	*4-OH-MPB*
5*	79.0 ± 4.1	66.2 ± 2.0
10*	78.1 ± 3.4	68.5 ± 1.6
20*	82.9 ± 1.7	68.2 ± 1.5
5**	94.3 ± 2.8	95.9 ± 4.5
10**	89.4 ± 2.7	92.4 ± 1.7
20**	92.3 ± 3.9	92.4 ± 1.7

* Plasma.
** urine.

TABLE II

ASSAY PRECISION FOR MPB AND 4-OH-MPB IN PLASMA

$n = 4$.

	MPB (μg/ml)			*4-OH-MPB (μg/ml)*		
	5	*10*	*20*	*5*	*10*	*20*
Mean observed concentration	4.86	9.91	19.79	4.81	10.01	20.05
Standard deviation	0.28	0.18	0.35	0.18	0.20	0.35
Coefficient of variation (%)	5.8	1.8	1.7	3.7	2.0	1.6

TABLE III

ASSAY PRECISION FOR MPB AND 4-OH-MPB IN URINE

$n = 4$.

	MPB (μg/ml)			*4-OH-MPB (μg/ml)*		
	10	*20*	*50*	*10*	*20*	*50*
Mean observed concentration	10.04	19.96	48.77	9.87	20.07	49.57
Standard deviation	0.20	0.07	0.47	0.18	0.10	1.05
Coefficient of variation (%)	2.0	0.3	1.0	1.9	0.5	2.1

terminations (Tables II and III) show that, exept for the lowest MPB and 4-OH-MPB plasma concentrations, the estimates of the drug concentration are highly reproducible. The precision of the method is well within acceptable limits for both compounds over the concentration range investigated.

Concerning the stability of MPB, it should be noted that the use of a 1% (w/v) ascorbic acid solution in which the internal standard is dissolved was sufficient to prevent the oxidation of MPB to 4-OH-MPB, at least during the extraction and evaporation step. Furthermore, by redissolving the residue only immediately before chromatographing the sample, further decomposition was avoided. When not to be analyzed, dry residues could be stored deep-frozen for at least 12 h without any degradation. Antioxidants should be added when blood and urine samples are collected.

To minimize chromatographic interferences due to phenolic and other amphoteric compounds[10–12], screening methods for non-steroidal anti-inflammatory drugs (NSAIDs), especially for doping analysis in horses, often use a washing of the organic layer with 0.1 *M* sodium bicarbonate. However, such a washing of the organic layer containing MPB resulted in a complete transfer of MPB from the organic to the aqueous phase and could consequently give rise to false negatives.

CONCLUSIONS

An HPLC method for the quantitation of MPB and its metabolite 4-OH-MPB in plasma and urine has been validated for concentrations ranging from 1 to 25 μg/ml in plasma and from 5 to 100 μg/ml in urine. The method is rapid, precise and accurate. It is currently applied to plasma and urine samples from horses after intravenous and oral administration of 8.8 mg/kg MPB.

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