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Determination of aditoprim and its oxidative metabolites in plasma and microsomal incubation mixtures by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method is presented for the determination of aditoprim (ADP) and its two oxidative metabolites in biological fluids including sheep plasma and the incubation medium of liver microsomes. The compounds were separated using a highly deactivated C_{18} column; the mobile phase consisted of an 0.05 *M* phosphate buffer (pH 6) and acetonitrile in a ratio of 90:10 (v/v). The eluate was quantified by ultraviolet detection at 230 nm. Calibration curves were linear from 0.25 to 5 μg/ml. The limit of detection was 0.05 μg/ml. In an *in vivo* kinetic study in sheep, N-monodesmethyl-ADP and N-didesmethyl-ADP appeared to be equally important metabolites. In contrast, in *in vitro* metabolism studies using liver microsomes from different animal species including sheep, N-didesmethyl-ADP was formed predominantly.

INTRODUCTION

Aditoprim (ADP), 2,4-diamino-5-[4-(dimethylamino)-3,5-dimethoxybenzyl]pyrimidine, is a relatively new dihydrofolate reductase inhibitor. Its chemical structure is closely related to that of trimethoprim (TMP), a bacteriostatic drug that is extensively used in human and veterinary pharmacotherapy. The antibacterial spectrum of aditoprim is similar to that of TMP but it exhibits different pharmacokinetic properties in farm animals [1–4].

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Two N-dealkylated metabolites resulting from ADP oxidation are known: N-monodesmethyl-ADP and N-didesmethyl-ADP (Fig. 1). One high-performance liquid chromatographic (HPLC) method [5] for the analysis of ADP has been published, but this method did not include its metabolites. Moreover, the eluent described for this system consisted of acetonitrile and a pH 9 buffer, and regular silica-based columns would rapidly degenerate at this elevated pH.

The aim of the present study was to develop a simple and sensitive method for the routine determination of both ADP and its two oxidative metabolites in biological samples. The method was applied to the analysis of plasma samples from *in vivo* pharmacokinetic studies in sheep and to the

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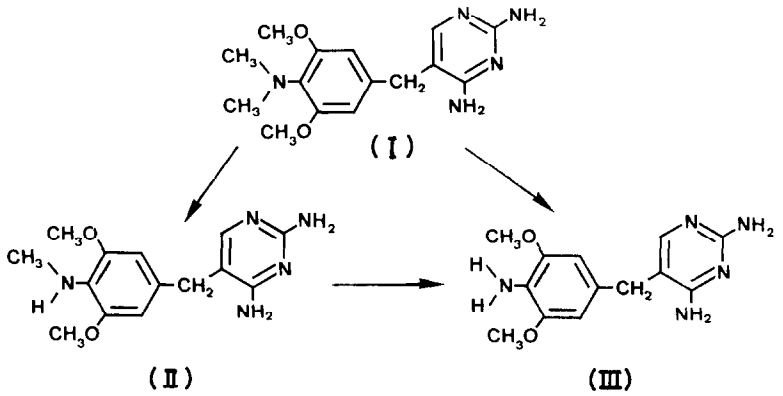

Fig. 1. Oxidative metabolism of aditoprim. I = Aditoprim; II = N-monodesmethyladitoprim; III = N-didesmethyladitoprim.

analysis of incubation media used in *in vitro* drug metabolism studies using liver microsomes of different species.

EXPERIMENTAL

Chemicals and reagents

ADP and its metabolites were kindly provided by Drs. W. F. Rehm and T. A. Graser (Hoffmann La Roche, Basle, Switzerland). TMP was obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Acetonitrile, ethyl acetate and dichloromethane originated from Farmitalia Carbo Erba (Milan, Italy); water was deionized and distilled; KH_2PO_4, Na_2HPO_4, H_3BO_3, KCl (all p.a. grade) were obtained from Merck (Darmstadt, Germany).

The borate buffer was prepared from 6.18 g of boric acid and 7.46 g of potassium chloride, which were dissolved in a minimum of distilled water. The pH was adjusted to 9 with *ca.* 30 ml of a 1 *M* sodium hydroxide solution. The resulting mixture was then diluted to 200 ml.

Apparatus

A constant-flow high-performance liquid chromatograph (Waters Assoc., Milford, MA, USA) consisting of a Model M6000 pump, a WISP 10B automatic injector and a Model M481 UV detector was connected to an LCI 100 laboratory computing integrator (Perkin Elmer, Norwalk, CT, USA). The column (250 mm × 4.6 mm I.D.) was packed with Supelcosil LC ABZ, 5 μm (Supelco, Paris, France) and protected by a 20 mm × 4.6 mm I.D. Supelco guard column packed with the same phase.

Standards

The solutions of ADP, N-monodesmethyl-ADP and N-didesmethyl-ADP used to construct the standards curves were prepared by serial dilutions of a stock solution (1.0 mg/ml in ethanol) in the mobile phase. Both the stock solution and the serial dilution were stable for at least three months at 0–4°C.

The internal standard (I.S.), TMP, was dissolved in a small amount of methanol and then diluted with borate buffer pH 9 so that the final concentration was 10 μg per ml of buffer.

Operating conditions

The mobile phase consisted of an 0.05 *M* phosphate buffer (pH 6) and acetonitrile (90:10, v/v); it was filtered over a 0.45-μm filter (Millipore) and degassed under vacuum before use. The flow-rate amounted 1.0 ml/min at a pressure of 100 bar. Analyses were performed at ambient temperature. The UV detector was set at 230 nm.

Drug disposition study

In order to test the application of the method in *in vivo* pharmacokinetic studies, the parent drug was administered intravenously to a sheep at a dose rate of 10 mg/kg. Blood was withdrawn

via the jugular vein at 5, 10, 20, 30, 45 and 60 min and at 2, 4, 6, 8, 12, 24 and 30 h after injection into heparinized tubes. The plasma was separated immediately and stored at −20°C until analysis.

In vitro metabolic study

Microsomes were prepared by differential ultracentrifugation [6] of homogenized liver fragments of rats, rabbits, cow, sheep, pigs, dogs and horses.

Enzyme activity was assessed by the level of N-monodesmethyl-ADP and N-didesmethyl-ADP formed. In a final volume of 1 ml of 0.1 *M* phosphate buffer (pH 7.4) a typical reaction mixture contained 0.8 μmol of NADPH, 1 mg of microsomal protein and 5 μmol of ADP.

Protein was determined according to the method of Lowry *et al.* [7]. Incubations were performed in glass vials at 37°C for 30 min. Termination of the reaction was achieved by the addition of 1 ml of extraction solvent (dichloromethane–ethyl acetate, 75:25, v/v).

Sample preparation

Plasma or microsomal incubations (100 μl) were mixed with 10 μl of the working solution of the internal standard (equivalent to 0.1 μg of TMP), 250 μl of borate buffer (pH 9) and 1.5 ml of extraction solvent (mixture of dichloromethane and ethyl acetate, 75:25, v/v) in a 2.0-ml polypropylene tube. The samples were shaken in a tabletop shaker for 20 min and centrifuged for 2 min at 5000 *g*. The organic layer was aspirated off, transferred to a 12-ml centrifuge tube and evaporated to dryness under a stream of nitrogen gas to prevent oxidation. The sample extract was reconstituted with 100 μl of the eluent and was injected onto the HPLC column.

Calibration and quality control

Standard calibration curves for ADP and metabolites in the range 0.25–5 μg/ml were prepared using sheep drug-free serum and 0.1 μg of TMP as the internal standard. Pooled serum samples were run through the procedure, and calibration curves were constructed using the analyte/internal standard peak-area ratio, calculated as a function of analyte concentration. Least-squares regression analysis was used to determine the slope.

The extraction efficiency of all the products was measured by comparing the peak area from the analysis of serum sample that were spiked with ADP and metabolites with the peak area resulting from a direct injection.

The intra-assay precision of the extraction procedure and chromatography was evaluated by processing as replicates on different days aliquots of pooled sheep serum samples containing known amounts of ADP and metabolites. The within-day precision of the assay was established by the repeated analysis (eight times) of samples spiked with ADP and its metabolites (1 μg/ml).

RESULTS AND DISCUSSION

Chromatography

Using the described chromatographic conditions, ADP, its metabolites and the internal stan-

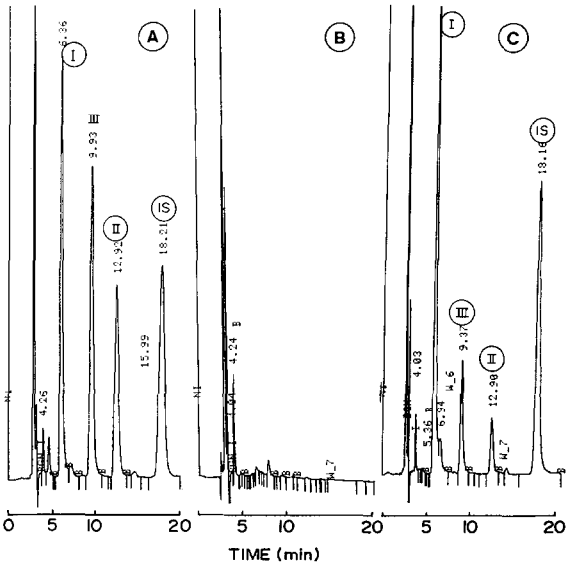

Fig. 2. (A) Chromatogram of a mixture of 100 ng each of aditoprim, N-monodesmethyladitoprim, N-didesmethyladitoprim and trimethoprim. (B) Chromatogram of blank sheep plasma. (C) Chromatogram of plasma sample from an animal 3 h after treatment with aditoprim (10 mg/kg intravenously). Peaks: I = aditoprim, 0.995 μg/ml; II = monodesmethyladitoprim, 0.226 μg/ml; III = didesmethyladitoprim, 0.288 μg/ml; IS = trimethoprim, 1 μg/ml.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF THE COMPOUNDS

Compound	Retention time (min)	Capacity factor	Asymmetry factor
ADP	6.36	1.10	1.04
N-Didesmethyl-ADP	9.93	2.31	1.07
N-Monodesmethyl-ADP	12.92	3.33	1.08
TMP	18.21	5.14	1.01

dard were well resolved. A typical chromatogram is shown in Fig. 2A. The retention times, capacity factors and asymmetry factor are reported in Table I. Because basic compounds are partially or completely ionized and often retained by residual silanol groups or the silica, this dual retention could reduce the efficiency and produce asymmetric peaks. Several ways to improve peak symmetry of basic compounds are known [8–10]. These include adding an ion-pair reagent or a competing base to the mobile phase, operating the column at high pH in combination with a silica saturator column and using a non-silica based packing. Each approach has some drawbacks [11].

In this case, within 18 min, a good separation was obtained, without any tailing peaks, by using a highly deactivated column for basic compounds (Supelcosil LC-ABZ). Blank samples from sheep plasma and microsomal incubations yielded straight baselines with no interfering peaks (Fig. 2B). Analysis of plasma spiked with standards (Fig. 2C) yielded sharp and fully separated peaks for ADP and its two oxidative metabolites.

TMP, a structural analogue of ADP, was chosen as the internal standard; it elutes after ADP and its metabolites. Moreover in our study, no other peaks occurred in this area.

Recovery

According to other authors [5] biological samples should be buffered at pH 9, to maximize the extraction recoveries of the compounds and to prevent possible degradation. The extraction recoveries of aditoprim, N-Didesmethyl-ADP and N-monodesmethyl-ADP were 80.7, 81.0 and 77.4% with relative standard deviations of 6.3, 4.6 and 5.1%, respectively.

Linearity

A linear correlation occurred between the peak-area ratio of ADP and metabolites to TMP. The concentration of the compounds was found to be in the range 0.25–5 μg/ml of plasma (Table II). The correlation coefficient generally exceeded 0.990 and the intercept did not significantly differ from zero.

Precision

As shown in Tables III and IV the reproducibility of the method was demonstrated by the value of the coefficient of variation (C.V.), which ranged from 2.47 to 6.32% for inter-assay and from 1.49 to 2.18% for intra-day studies.

Detection limits

The limit of detection of the assay is defined as

TABLE II

LEAST-SQUARES REGRESSION STATISTICS FOR HPLC CALIBRATION DATA OF ADITOPRIM AND ITS METABOLITES IN SPIKED SAMPLES OF SHEEP PLASMA, USING TRIMETHOPRIM AS INTERNAL STANDARD (n = 5)

Compound	Slope (mean ± S.D.)	Intercept	Correlation coefficient
ADP	0.861 ± 0.010	0.011	0.995
N-Didesmethyl-ADP	0.894 ± 0.007	0.007	0.997
N-Monodesmethyl-ADP	0.655 ± 0.006	0.015	0.997

TABLE III

INTER-ASSAY PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF ADITOPRIM AND ITS METABOLITES (n = 5)

Values in parentheses are coefficients of variation (%).

Concentration (μg/ml)	Concentration found (μg/ml)		
	ADP	N-Didesmethyl-ADP	N-Monodesmethyl-ADP
0.25	0.229 ± 0.012 (5.48)	0.239 ± 0.012 (5.39)	0.252 ± 0.015 (6.32)
0.50	0.482 ± 0.018 (3.82)	0.484 ± 0.012 (2.47)	0.491 ± 0.013 (2.76)
1.00	1.012 ± 0.061 (6.01)	1.013 ± 0.053 (5.29)	0.989 ± 0.059 (6.04)
2.50	2.521 ± 0.084 (3.35)	2.505 ± 0.071 (2.84)	2.491 ± 0.098 (3.96)
5.00	4.988 ± 0.283 (5.67)	4.995 ± 0.192 (3.86)	5.008 ± 0.193 (3.85)

the amount of drug that resulted in a peak area three times that of the baseline noise in a blank sample. It was found to be 0.05 μg/ml for ADP and its two desmethylated metabolites.

Preliminary kinetic study

The kinetics of plasma concentrations in ADP and its two metabolites is illustrated in Fig. 3. In accordance with results from others [1], the parent drug decreased rapidly during the first 2 h, whereas the two desmethylated metabolites were present in the plasma of sheep, probably because of extensive liver metabolism of the drug.

Application in metabolism study

The *in vitro* biotransformation of aditoprim was measured in liver microsomal fractions prepared from various animal species (Fig. 4). In all investigated species, ADP was converted into its two oxidative derivatives. N-Didesmethyl-ADP was by far the major metabolite and its formation rate varied from 5 (rat) to 35 (horse) nmol/mg protein/min. In sheep the ratio of didesmethylated to monodesmethylated derivatives was about 5:1. This result is in good agreement with that obtained in the first plasma samples of the *in vivo* pharmacokinetic study. By contrast, from 2

TABLE IV

INTRA-DAY PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF ADITOPRIM AND ITS METABOLITES (n = 8)

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)
ADP	1.00	1.024 ± 0.020	1.97
N-Didesmethyl-ADP	1.00	1.011 ± 0.015	1.49
N-Monodesmethyl-ADP	1.00	1.037 ± 0.022	2.18

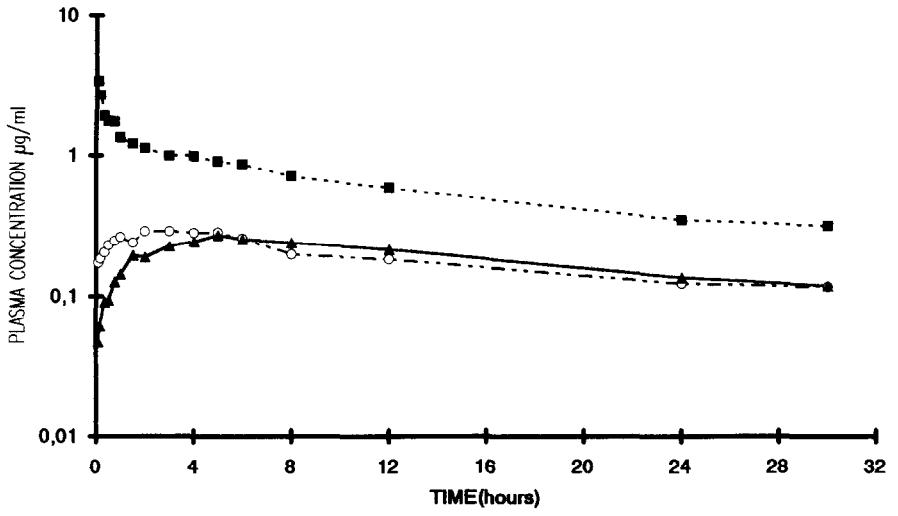

Fig. 3. Plasma concentration–time curve in a sheep given a 10 mg/kg intravenous dose of aditoprim. ■, Aditoprim; ▲, N-monodesmethyladitoprim; ○, N-didesmethyladitoprim.

h after the administration and thereafter, this value was around 1:1. This could be because of elimination or *in vivo* catabolism of N-didesmethyl-ADP, which take place progressively in animals and which could not occur under the conditions of the *in vitro* incubation experiments.

CONCLUSION

This HPLC method appears to have an excellent potential for determining ADP and its main metabolites in biological samples including plasma and *in vitro* incubation medium. Advantages include the simplicity, specificity and reproducibility of this technique when compared with a previously published method [5]. The use of an eluent pH 6 buffer strongly increases column lifetime.

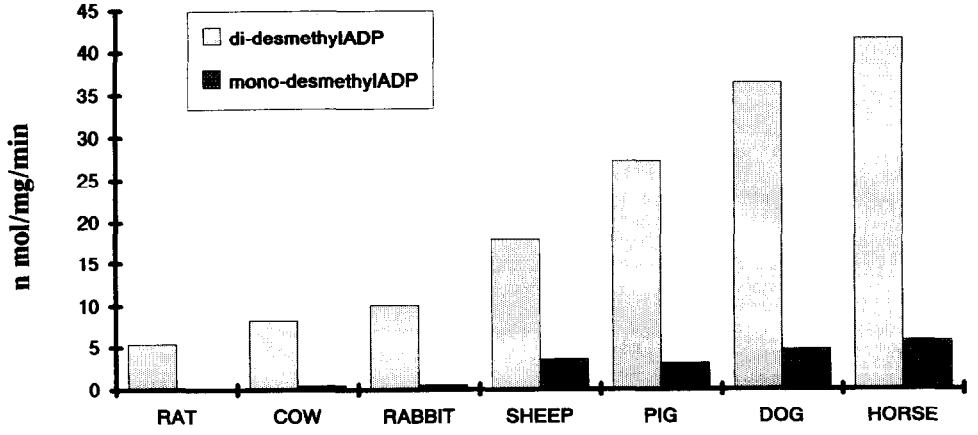

Fig. 4. *In vitro* biotransformation of aditoprim measured in liver microsomal fractions prepared from various animal species (5 µmol of aditoprim per mg of microsomal protein).

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