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DETERMINATION OF ADITOPRIM, A NEW DIHYDROFOLATE REDUCTASE INHIBITOR, IN THE PLASMA OF COWS AND PIGS

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SUMMARY

A selective and sensitive high-performance liquid chromatographic method was developed for the determination of aditoprim, a new dihydrofolate reductase inhibitor, in the plasma of cows and pigs. The compound and its internal standard were extracted with chloroform from plasma buffered at pH 9 and chromatographed on a μBondapak reversed-phase column with a mixture of acetonitrile and 0.5% ammonium carbonate aqueous solution and detected by UV absorption. Aditoprim can be quantitatively extracted from plasma. The limit for quantitative determination was ca. 0.050 μg/ml with a standard deviation of ± 0.006 μg/ml and an accuracy of ± 3%. The assay was linear over the concentration range 0.1—5.0 μg/ml (precision 0.007—0.09 μg/ml), and the day-to-day accuracy was better than ± 3.5%. No interference was observed from either metabolite(s) or coadministered sulphonamides. The new procedure was compared with a microbiological assay by analysing plasma samples from pigs treated with aditoprim. The two methods gave similar results in the range 0.5—5.0 μg/ml.

INTRODUCTION

Aditoprim, 2,4-diamino-5-[4-(dimethylamino)-3,5-dimethoxybenzyl]-pyrimidine, is a new dihydrofolate reductase (DHFR) inhibitor. Its chemical structure (Fig. 1) is close to that of trimethoprim (TMP). It is a base, and is somewhat more lipophilic than TMP, with pK_a values of 7.8 and 6.7. The antibacterial spectrum of aditoprim is similar to that of TMP, but it exhibits different pharmacokinetic properties in farm animals, which could enable its use as a single drug or as a combination with sulphonamide [1].

In recent years, high-performance liquid chromatography (HPLC) has proved to be a powerful technique for the analysis of TMP in biological fluids [2—7],

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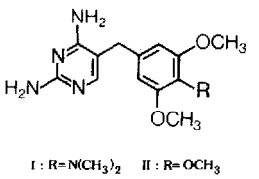

I : R= $N(CH_3)_2$ II : R= OCH_3

Fig. 1. Chemical structures of aditoprim (I) and trimethoprim (II).

thus enabling pharmacokinetic studies to be carried out in man or animals. HPLC has several advantages over microbiological assay, such as shorter analysis time, greater specificity and limit of quantification.

This paper describes a reversed-phase HPLC method, with UV detection, for the determination of aditoprim in the plasma of cows and pigs, and its application to the study of the pharmacokinetics of the new drug in farm animals.

EXPERIMENTAL

Reagents, solvents and materials

All chemicals and solvents were p.a. quality. Water was distilled in an all-glass apparatus. Chloroform, boric acid, diammonium hydrogen phosphate, potassium chloride and sodium hydroxide were obtained from Merck (Darmstadt, F.R.G.) and HPLC-grade acetonitrile from Rathburn (Walkerburn, U.K.). Chloroform was distilled before use. Ammonium carbonate was purchased from Fluka (Buchs, Switzerland).

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 by ultrasonification. 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 in a volumetric flask.

Aditoprim used for the preparation of the standard or for quality control was supplied by Dr. I. Kompis (F. Hoffmann-La Roche). The internal standard was 2,4-diamino-5-(4-bromo-3,5-dimethoxybenzyl)-pyrimidine (brodimoprim, F. Hoffmann-La Roche). Blank plasma of cows and pigs was obtained from the slaughterhouse of Basel (Switzerland).

Plasma standard and quality test solutions

Five aqueous working solutions were derived from a stock solution containing 50 mg of aditoprim dissolved in 50 ml of methanol, by diluting 10, 5, 2 and 1 ml, respectively, with doubly distilled water to 100 ml, and 0.5 ml to 250 ml in a volumetric flask. These solutions were named a, b, c, d and e. The plasma standards were prepared by diluting 1 ml of a, c, d and e to 20 ml, and 1 ml of b to 25 ml with blank plasma. The concentrations of the standards were 5, 2, 1, 0.5 and 0.1 μg/ml aditoprim, respectively. Another set of standards was derived from the 0.5 μg/ml plasma solution, and contained 0.25 and 0.05 μg/ml aditoprim.

Various plasma samples of known aditoprim concentrations were prepared from another stock solution and used as quality-control samples in order to check the accuracy and precision of the method. To obtain optimum control

of the assay, plasma standards and quality-control samples were prepared by different analysts, using different stock solutions.

Both the stock solution and the plasma standards were stable for at least two weeks at 0—4°C. The internal standard (brodimoprim) was dissolved in a small amount of methanol, and then diluted to 100 ml with water so that the final concentration was 4 μg per 100 μl water. The standard and the quality-control samples were deep frozen at −20°C for at least 24 h. Before analysis, the samples [plasma standard (calibration), control sample (quality control), biological sample (analyses) and drug-free plasma (blank plasma)] were allowed to thaw at 37°C and were then homogenized using a rotating shaker (5 min).

Chromatographic system

The HPLC system consisted of an LC 414 pump (Kontron, Zürich, Switzerland), a variable-wavelength LC—UV detector (Pye Unicam, Cambridge, U.K.) operating at 290 nm, an SP 4100 integrator (Spectra-Physics, Basle, Switzerland), and an automatic injector Model ASI 45 (Kontron), which was modified in order to allow the use of 3-ml micro-reaction vessels from Supelco (Bellefonte, PA, U.S.A.). With this modification, the instrument could inject a 100-μl aliquot from a 200-μl sample. The stainless-steel column was either 250 mm × 4.6 mm I.D. containing 10-μm LiChrosorb RP-18 (Merck) or 300 mm × 3.4 mm I.D. containing 7-μm μBondapak (Waters Assoc., Milford, MA, U.S.A.). The isocratic mobile phase was acetonitrile—0.5% aqueous ammonium carbonate solution (pH 9) or acetonitrile—0.6% diammonium hydrogen phosphate solution (pH 8) in a 3:7 (v/v) ratio. A guard column filled with either LiChrosorb RP-18 or μBondapak was inserted between the pump and the injector in order to prevent fast degradation of the analytical column by the alkaline mobile phase. The eluent was degassed under vacuum for ca. 30 s.

Procedure

Extraction. A 1-ml aliquot of the biological sample was measured into a ground-glass-stoppered centrifuge tube and mixed with 50 μl of internal standard (2 μg). Borate buffer (1 ml, pH 9) was added, followed by 4 ml of chloroform. The sample was extracted by shaking for 5 min on a reciprocating shaker (rate, 80 rpm) and centrifuged at 1760 *g* for 10 min. The aqueous phase was discarded by suction; an aliquot (ca. 3 ml) of the organic phase was transferred to the conical micro-reaction tube (Supelco) and evaporated to dryness at 43°C under a gentle stream of pure nitrogen.

Chromatography. The residue from the extraction was reconstituted with 200 μl of mobile phase, and 100 μl of the clear solution were injected for HPLC analysis. The flow-rate of the mobile phase was 1.7 ml/min and the column was operated at room temperature (ca. 21°C). Under these conditions, the retention times of aditoprim and the internal standard were ca. 4 and 5.5 min (LiChrosorb RP-18) or ca. 4 and 7 min (μBondapak), respectively.

Calibration and quality control. Together with the unknown samples, the corresponding calibration samples, each consisting of five plasma standards with the appropriate concentration of aditoprim, were processed as described above. The calibration curves were obtained by least-squares regression of the

peak-height ratio of aditoprim to internal standard versus the concentration of aditoprim. These curves were then used to interpolate the concentrations of aditoprim from the respective peak-height ratios obtained from the unknown samples. All data processing and calculations were carried out on the Spectra-Physics SP 4100 computing integrator and the Minifile 4100D [8].

RESULTS AND DISCUSSION

Specificity and choice of the internal standard

The method was developed for the determination of aditoprim in plasma of cows and pigs. Several blank samples from different cows and pigs were analysed as described above. In all cases, clean plasma extracts were obtained, indicating specificity of the assay with respect to other endogenous components in the plasma (Figs. 2—4). In addition, plasma samples from cows and pigs which had received intravenous or intramuscular doses of aditoprim were analysed without adding the internal standard. In no case was interference of co-extracted metabolites observed with the internal standard. Sulphamerazine and sulphadimidine eluted within the first few minutes and did not interfere with either aditoprim or the internal standard. This was shown by analysing plasma from cows which had received both the sulphonamide and aditoprim (Fig. 3).

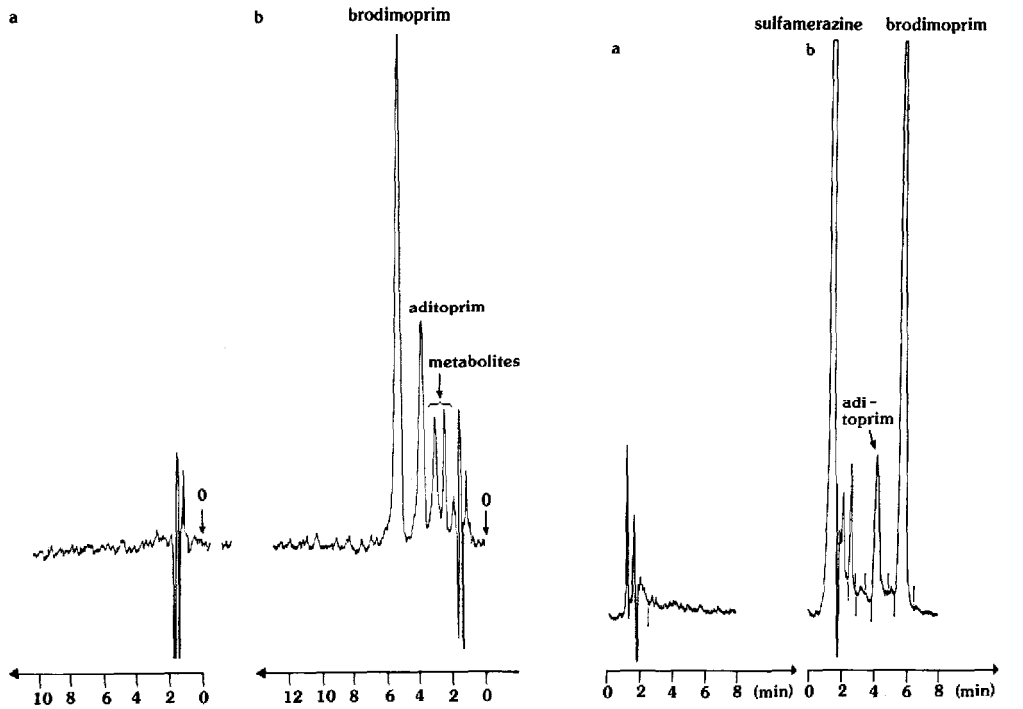

Fig. 2. Chromatograms of extracts of pig plasma. (a) Blank plasma; (b) biological sample (aditoprim, 0.500 μg/ml; brodimoprim, 2.0 μg/ml). Column, 10 μm LiChrosorb RP-18 (250 × 4.6 mm I.D.).

Fig. 3. Chromatograms of extracts from plasma of a cow treated with the combination sulphamerazine/aditoprim. (a) Blank plasma; (b) biological sample (aditoprim, 0.4 μg/ml; brodimoprim, 2 μg/ml). Chromatographic conditions as in Fig. 2.

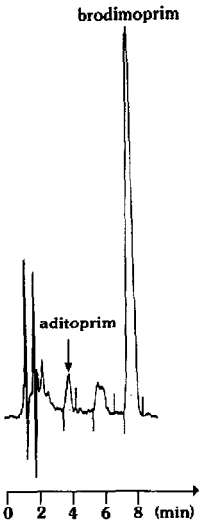

Fig. 4. Chromatograms of extracts from pig plasma fortified with aditoprim (0.05 μg/ml) and brodimoprim (1 μg/ml). Column, 7 μm μBondapak (300 × 3.4 mm I.D.).

A 10-μm LiChrosorb RP-18 or a 7-μm μBondapak phase were utilized during routine analysis. However, the separation between aditoprim and the internal standard was better with μBondapak than with LiChrosorb (Figs. 2 and 4). The peak symmetry was optimized by buffering the mobile phase either to pH 9 with ammonium carbonate or to pH 8 with diammonium hydrogen phosphate (Merck). With a potassium phosphate buffer, strong tailing, or even no elution of aditoprim, was observed whatever the pH selected.

Limit for quantitative determination

The limit of quantitation of the new assay, defined as the minimum concentration that could be measured with an accuracy better than 10% [9—11], was found to be 0.050 μg/ml aditoprim in plasma with a standard deviation of ± 0.006 μg/ml and an accuracy of ± 3%. The intra-assay precision of the limit of quantitation was determined by analysing on the same day four samples fortified with 0.050 μg/ml aditoprim with the detector sensitivity set at 0.01 a.u.f.s.

Linearity

A linear correlation between the peak-height ratio of aditoprim to brodimoprim and the concentration of aditoprim was found in the range 0.1—5 μg/ml of plasma. The coefficient of determination was generally better than 0.9990 and the intercept did not differ significantly from zero ($p > 0.05$). For lower concentrations, it was necessary to calibrate over the range 0.05—0.5 μg aditoprim per ml plasma.

Recovery

The plasma samples have to be buffered to pH 9 for the extraction of the drug; in the biological samples, higher pH values may lead to the conversion into the parent drug of possible N-oxide metabolites of aditoprim. The

recovery (extraction yield) was determined from the difference between the peak height when aditoprim was added to plasma, and the peak height when it was added to the final extract of a blank plasma. The extraction yield of aditoprim was ca. 100% in the concentration range 0.5—2.5 μg/ml of plasma. The same was true for the internal standard brodimoprim.

Accuracy and precision

The intra-assay and the inter-assay reproducibilities were determined by two analysts using the same apparatus. The intra-assay reproducibility was determined by analysing five to nine samples at various concentrations on one day. The inter-assay variability [12] was obtained by assaying biological samples over two months; the number of replicates in each concentration was between 7 and 14. As shown in Tables I and II, the intra-day and inter-day variability and precision of the new HPLC method were acceptable over the investigated concentration range.

TABLE I

INTRA-ASSAY PRECISION AND ACCURACY

Sample	Added (μg/ml)	Found (mean ± S.D.) (μg/ml)	Accuracy (found − added) (%)	95% Confidence limit (μg/ml)	Replicates
Pig plasma	0.050	0.051 ± 0.006	+2	0.047—0.056	9
Cow plasma	0.450	0.446 ± 0.013	−1	0.435—0.457	8
Pig plasma	1.13	1.13 ± 0.02	<0.5	1.10 −1.17	5
Pig plasma	2.26	2.34 ± 0.03	+3.5	2.31 −2.36	6

TABLE II

INTER-ASSAY PRECISION AND ACCURACY OBTAINED FROM THE ANALYSIS OF COW PLASMA

Added (μg/ml)	Found (mean ± S.D.) (μg/ml)	Accuracy (found − added) (%)	95% Confidence limit (μg/ml)	Replicates
0.111	0.112 ± 0.007	+0.9	0.106—0.118	8
0.120	0.119 ± 0.008	−0.8	0.114—0.123	14
0.556	0.544 ± 0.016	−2.2	0.530—0.557	8
0.599	0.587 ± 0.021	−2.0	0.575—0.599	14
1.11	1.10 ± 0.01	−0.9	1.09 −1.11	7
1.20	1.18 ± 0.04	−1.7	1.16 −1.2	14

Stability of aditoprim in cow plasma

Aditoprim was added to blank plasma at three different concentrations (0.25, 5.0 and 10 μg/ml) and stored at different temperatures for different time intervals (three months at −20°C, one day at room temperature). A set of seven freshly prepared control samples was analysed, together with five stored samples of the same concentrations. The data processing was carried out according to Timm et al. [13]. Table III shows that in no case was there a

TABLE III

STABILITY OF ADITOPRIM IN SPIKED COW PLASMA UNDER VARIOUS STORAGE CONDITIONS

Concentration of aditoprim (μg/ml)	Storage conditions	Difference (found − added) (%)	90% Confidence interval (%)	
			Lower limit	Upper limit
0.250	24 h, room temperature	+1.5	−2.6	+5.8
	three months, −20°C	−1.4	−5.8	+3.2
5.00	24 h, room temperature	+1.8	−1.0	+4.8
	three months, −20°C	+3.4	+0.6	+6.3
10.0	24 h, room temperature	−0.9	−3.6	+1.8
	three months, −20°C	−2.5	−4.5	−0.5

significant difference between the results from stored and control samples.

Application of the method to biological samples

The method was used to analyse pig plasma following single intramuscular doses of 5 mg aditoprim per kg body-weight. Table IV shows the results obtained from one pig. The analyses were carried out in duplicate on two different days. The samples were also analysed using a microbiological method [14]. The results obtained with the microbiological method were plotted against those from HPLC. A straight line could be fitted to these results by means of a computer program of linear regression analysis, both variables being subjected with error [15].

The value of the slope of the line did not differ significantly from 1 (95%

TABLE IV

PLASMA CONCENTRATIONS OF ADITOPRIM IN PIG, MEASURED WITH A MICROBIOLOGICAL METHOD (A) AND WITH THE NEW HPLC TECHNIQUE (B)

A single intramuscular injection of a dose of 5 mg/kg body weight.

Time after injection (h)	Aditoprim concentration (μg/ml)	
	A	B
0.33	3.02	2.93
0.62	2.42	2.46
1.0	2.17	2.06
1.5	1.74	1.74
2.0	1.91	1.72
3.1	1.57	1.50
5.0	1.30	1.27
6.0	1.30	1.11
7.0	1.04	1.07
23.0	0.56	0.450
24.0	0.51	0.424
25.0	—	0.405

confidence limits: 0.9599—1.0033). However, the intercept differed from zero, since the 95% confidence limits were 0.071—0.127 μg/ml. Nevertheless, these values were lower than the limit for quantitation of the microbiological method (0.5 μg/ml). Therefore, in the concentration range 0.5—5 μg/ml, the two analytical methods gave similar results ($p > 0.05$).

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