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Short communication

Determination of indomethacin residues in poultry by high-performance liquid chromatography

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Abstract

A HPLC method using a C_{18} column and UV detection (254 nm) is described for the determination of indomethacin residues in chicken tissues (liver, muscle and fat). Drug extraction from tissue homogenate in phosphate buffer (pH 3.5) was performed with dichloromethane. Mobile phase was acetonitrile–acetic acid (0.5% in water) (50:50). Indomethacin detection limit was 20 ng/g for the studied tissues. After administration of an oral dose of indomethacin (2 mg/kg), only three of the eight poultry studied showed drug tissue levels, in those cases the levels were below 50 ng/g.

Keywords: Indomethacin

1. Introduction

Indomethacin is a non-steroidal antinflammatory (NSAID) and antipyretic drug used in man, useful for the relief of symptoms of rheumatoid arthritis [1]. In veterinary medicine, it is effective in treatment of inflammatory processes related to infectious disease. The drug is usually administered orally together with drinking water. Pharmacokinetic studies of intravenous-administered indomethacin in cattle show a wide extravascular distribution as suggested by the high volume of distribution and the long elimination half-life [2]. Similar kinetic behaviour of indomethacin has been observed after intramuscular administration in sheep [3] and oral administration in poultry (in preparation). These data suggest that indomethacin achieves high tissue levels.

Nowadays, health authorities have increased their requirements concerning drug residues in edible tissues. It is important to develop proper techniques to isolate and quantify drugs in tissues of food-producing animals.

Some methods have been described for indomethacin determination in biological fluids. Berstein and Evans [4] described a high-performance liquid chromatography (HPLC)–fluorescence method for determination of indomethacin in urine, and Al-Angary et al. [5] used a HPLC–UV spectrometry technique to determine indomethacin in plasma achieving a quantification limit of 50 ng/ml.

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The aim of the present study was to develop a method to isolate and quantify indomethacin in chicken liver, fat and muscle by HPLC. This method was also applied to the study of tissue residues in broiler chickens treated with an oral dose of indomethacin.

2. Experimental

2.1. Chemicals

Indomethacin was provided by JAER (St. Vicenç dels Horts, Spain). Suxibuzone base was obtained from Dr. Esteve (Barcelona, Spain). HPLC reagent grade acetonitrile, methanol and dichloromethane were purchased from Riedel-de Haën (Seelze, Germany). The other reagents were of analytical reagent grade. Water was double-distilled and deionized.

Standard solutions of indomethacin and suxibuzone were prepared by dissolving the drug in methanol at a concentration of 10 μg/ml. These solutions were stored in the dark at 4°C.

2.2. Chromatographic system

The HPLC system consisted of a Waters 717 autosampler injector, a Waters 481 UV detector and a Waters pump 600E multisolvent delivery system (Milford, MA, USA). Separation was achieved on a Waters Spherisorb ODS-2 column (200 mm×3.9 mm I.D., 5 μm). The mobile phase was acetonitrile–0.5% acetic acid (50:50, v/v) and the flow-rate was 1.5 ml/min. The chromatogram was monitored at a wavelength of 254 nm throughout the analysis. Analyses were carried out at room temperature (20°C) and data-processing was handled by a Waters 746 Data Module.

2.3. Animals

Broiler chickens weighing 1.5 kg (±0.2), purchased from a poultry farm, were kept in environmentally controlled rooms, with the temperature maintained at 24±2°C and a light–dark cycle of 7:00–19:00 h for one week before administration of the medication. Clinical signs of disease were not apparent. Food and water were supplied ad libitum although 18 h before the experiments food was withdrawn.

Indomethacin was administered orally to 16 chickens at a dose of 2 mg/kg. Eight chickens were slaughtered by exsanguination in groups of four at 8 h and 24 h after treatment. The eight remaining animals were slaughtered three days after drug administration. Liver, muscle and fat samples were randomly collected and frozen at −20°C until processing.

In order to define method conditions, liver, fat and muscle samples were randomly removed from chickens obtained from a local market.

2.4. Sample preparation

Five grams of the different tissues were homogenized during 5 min, by ultraturrax, with 15 ml of a 0.25 *M* Na_2HPO_4 solution (pH 3.5). Four ml (1 g tissue) of homogeneous tissue mixture was transferred to a tube containing 500 ng of suxibuzone as internal standard. Samples were then extracted with 20 ml of dichloromethane by gently blending for 30 min. Tubes were centrifuged at 2000 *g* for 20 min to allow phase separation. Aqueous phase was discarded and the organic phase was evaporated until dryness using a rotary evaporator. Liver and muscle extracts were redissolved with 200 μl of methanol. Fat sample extract, consisting of an oil residue, was mixed with 300 μl of methanol and the unstable emulsion obtained was allowed to stand for 10 min until the total phase separation occurred. Approximately 50 μl of each methanolic solution was injected into the chromatographic system. Blank tissue samples were prepared in a similar fashion except that no drugs were added.

2.5. Recovery

Drug-free tissue samples were spiked with standard indomethacin in the range 20–200 ng/g and processed by the described procedure (internal samples). After extraction, 500 ng of suxibuzone (internal standard) were added for analysis. A standard series of indomethacin samples containing internal standard was also prepared, with the same con-

centration range and was directly analyzed by HPLC without extraction (external samples). Extraction efficiencies were determined by comparison of HPLC results of internal and external samples.

2.6. Calibration

A standard series in the range 20–500 ng/g of indomethacin in drug-free tissue samples were prepared and processed. Method linearity, quantification limit, precision and accuracy were calculated. The limit of quantification was determined studying the accuracy and precision from samples containing 20, 50, 100, 200 and 500 ng/g of indomethacin. The limit of quantification represents the minimum concentration with an accuracy and precision within the established range.

3. Results and discussion

Fig. 1 shows typical chromatograms from a blank (left side) of the different chicken tissues and tissue samples spiked with indomethacin and suxibuzone (right side). The retention times were 6.4 min for indomethacin and 5.4 min for suxibuzone. Blank samples of the different tissues did not show any interfering substance in the retention time of indomethacin and suxibuzone. Therefore under the chromatographic conditions described, the indomethacin and internal standard peak are well reproduced for the three assayed tissues.

Method specificity can be demonstrated by comparison of chromatograms of blank samples and samples spiked with both drugs. Samples containing drugs presented chromatograms with a good resolution for indomethacin and suxibuzone.

Some HPLC methods have been employed to determine indomethacin in plasma and urine samples using reversed-phase columns [4,6,7]. However, under similar conditions, no data have been obtained about the analysis of residues in animal tissues. Although the above mentioned authors used similar mobile phases, columns (100 mm column length or 10 μm particle size) and wavelength, a change in solvent ratio was necessary to determine indomethacin in tissues. The 50:50 ratio of acetonitrile–0.5% acetic acid was ideal to isolate and quantify indomethacin and suxibuzone without interferences.

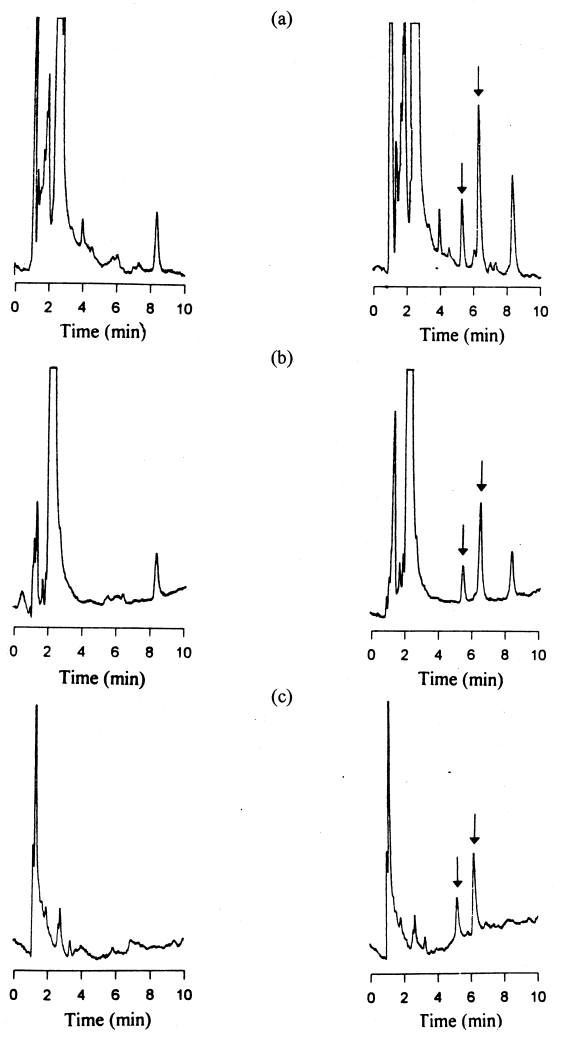

Fig. 1. Chromatograms of blank samples (left side) and samples spiked with 200 ng/g of indomethacin (6.4 min) and 200 ng/g suxibuzone (5.4 min) (right side) of liver (a), muscle (b) and fat (c).

3.1. Recovery

Table 1 shows the recovery of indomethacin obtained from liver, fat and muscle over the range 20–200 ng/g. In liver samples, the recoveries varied from 81 to 89%. Fat samples showed the lowest results with recoveries ranging from 63.1 to 88.8%,

Table 1
Recovery of indomethacin obtained in chicken liver, muscle and fat samples spiked with different concentrations of the drug

Spiked concentration (ng/g)	Recovery (%)		
	Liver	Muscle	Fat
20	88.8±6.9	100.0±4.2	88.8±7.3
50	89.0±14.9	100.0±7.1	64.0±4.7
100	81.1±7.2	99.3±4.3	69.1±3.6
200	85.3±8.2	98.6±0.1	63.1±2.0

Data are expressed as mean±S.D. (n=4).

due to the complex matrix composition and drug properties. Indomethacin is a strong lipophilic drug, thus making its extraction from fat samples difficult.

The best results were obtained from muscle affording values of 100% with a minimum of 98%.

The sample extraction conditions of indomethacin at a pH value of 3.5 eased and improved recovery of the drug from the different samples studied. Moreover, the quantity of sample selected for the analysis was the most suitable to achieve a good sensibility, recovery and specificity since the influence of the different substances present in the samples was lower.

3.2. Calibration

Tissue samples spiked with five different concentrations of indomethacin were analyzed. All analysis were performed in quintuplicate. The peak area ratios (indomethacin to suxibuzone as internal standard) were linearly related to the concentration over the range 20–500 ng/g. The equations for the straight lines were $y=4.797x+0.002$ ($r=0.9983$) for liver, $y=2.036x+0.022$ ($r=0.9955$) for fat and $y=4.321x+0.029$ ($r=0.9987$) for muscle samples, y being the peak-height ratio and x the indomethacin concentration (ng/g).

Precision of the method was expressed by comparing five calibration straight lines for intra-day variability and five more for inter-day assay. Reproducibility was determined in the indomethacin concentration range of 20 and 500 ng/g of tissue. Accuracy of the method could be measured by the differences between observed and calculated concentration results, and expressed as the relative error. Corresponding results for the three studied tissues are shown in Table 2. Precision results are acceptable according to Horwitz criterium [8].

The quantification limit for indomethacin was found to be 20 ng/g in all tissues analyzed. Quantification limits attained by other authors with a similar applied method to plasma and urine samples [5] were significatively higher than those obtained by us in liver, muscle and fat, and similar results were found by Berstein and Evans [4] in plasma and urine using a fluorescence detection.

Linearity, quantification limit, precision and accuracy were consistent with a reliable method with a good sensitivity over the studied range. This range sufficiently covers the indomethacin concentration to be expected in tissue samples of treated animals.

3.3. Study of tissue residues

Indomethacin administration in veterinary medicine is commonly related to antinflammatory therapy in small animals and horses. However, in European countries, this drug has been recently introduced as an efficient coadjutant therapy in infectious diseases in food-producing animals. There are few studies about this recent application and, especially, there is a lack of literature concerning indomethacin residues in edible tissues.

To test the method, tissues (liver, muscle and fat) from slaughtered chickens treated with a oral dose of 2 mg/kg of indomethacin were studied. Corresponding results are illustrated in Table 3. Indomethacin concentrations observed in the assayed tissues suggest a drug diffusion towards peripheric tissues. However 24 h after administration tissue, concentrations of indomethacin were found to be very low and even undetectable in some animals, thus suggesting a quick elimination of the drug. Results are in agreement with pharmacokinetic data observed in humans [1], rabbits [5] and cattle [2]. Three days after treatment only three of eight animals showed indomethacin residues over the quantification limit. For those animals the indomethacin levels were below 50 ng/g. This data suggest that three days after treatment the indomethacin tissue concentration would be not significant.

Table 2
Intra-day and inter-day precision and accuracy for indomethacin determination in liver, muscle and fat samples spiked with different concentrations of the drug

Tissue	Spiked concentration (ng/g)	Intra-day			Inter-day		
		Measured concentration (ng/g)	R.S.D. (%)	Accuracy (%)	Measured concentration (ng/g)	R.S.D. (%)	Accuracy (%)
Liver	20	21	17.2	5.0	23	21.5	15.0
	50	50	3.5	0.0	54	12.7	8.0
	100	98	1.0	2.0	95	3.3	5.0
	200	201	3.2	0.7	199	2.1	0.5
	500	500	0.5	0.1	501	0.4	0.2
Muscle	20	17	5.9	15.0	20	23.3	0.0
	50	51	2.3	1.3	50	7.0	0.0
	100	96	0.6	3.7	97	5.0	3.0
	200	196	0.8	0.2	203	3.4	1.5
	500	495	1.4	1.0	499	0.6	0.2
Fat	20	23	6.7	13.3	24	26.9	18.0
	50	46	12.1	8.7	51	14.3	2.0
	100	102	3.4	2.3	98	8.9	2.0
	200	198	1.0	0.8	204	3.2	2.0
	500	501	0.2	0.1	500	0.4	0.0

Data are expressed as mean±S.D. (n=5).

Table 3
Drug tissue concentrations (ng/g) in chickens given indomethacin orally (2 mg/kg)

Time after drug administration	Indomethacin concentration (ng/g)		
	Liver	Muscle	Fat
8 h	572	32	65
	265	39	ND
	287	44	29
	457	56	ND
24 h	27	33	72
	34	35	ND
	23	ND	ND
	ND	ND	ND
3 days	ND	25	29
	ND	ND	ND
	ND	ND	ND
	ND	ND	ND
	31	22	ND
	ND	ND	ND
	ND	26	20
	ND	ND	ND

ND=Not detected.

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