ELSEVIER

Journal of Chromatography B, 693 (1997) 489–492

JOURNAL OF CHROMATOGRAPHY B

Short communication

Rapid and sensitive determination of amprolium in chicken plasma by high-performance liquid chromatography with post-column reaction

K. Hamamoto[a,*], R. Koike[a], A. Shirakura[a], N. Sasaki[a], Y. Machida[b]

[a]*National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Tokura 1-15-1, Kokubunji-shi, Tokyo 185, Japan*

[b]*Department of Clinical Pharmacy, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan*

Received 5 November 1996; revised 14 January 1997; accepted 24 January 1997

Abstract

A rapid, sensitive and reproducible reversed-phase HPLC assay was developed for the determination of amprolium (APL) in chicken plasma. Protein in plasma sample was precipitated with 0.33 *M* perchloric acid and supernatant solution was injected into the HPLC system. Following the chromatographic separation of APL and the beclotiamine (I.S.) on a C_{18} column, the derivatives of APL and I.S. were formed by post-column reaction and detected by fluorescence detection (excitation at 400 nm, emission at 460 nm). The method showed excellent precision, accuracy and speed with a detection limit of 2 ng/ml. The intra- and inter-assay variance of this method were less than 11.2%. This method has been successfully applied to plasma determinations after oral administration of APL to chicken.

Keywords: Amprolium

1. Introduction

Amprolium (APL) has been used as a preventive and clinical anticoccidial drug in chicken. APL shows its effect mainly in the gastrointestinal tract, but it remains in eggs and other organs [1]. In addition, it has been known that APL and thiamine apparently compete for intestinal absorption in chicken [2]. In ruminants, polioencephalomaracia is induced by deficiency of thiamine in plasma after excessive APL administration [3,4]. So, we feel the bioavailability of APL is very interesting.

However the concentration of APL in plasma has not been determined accurately because of its very low therapeutic level. Some HPLC methods for the determination of APL in muscle or liver have been reported [5–7], but these methods could not be used for the determination of the concentration of APL in chicken, owing to the improper internal standard (I.S.) or lack of I.S. Therefore we tried to develop a simple and sensitive determination method for APL

*Corresponding author.

PII S0378-4347(97)00088-1

in chicken plasma by post-column HPLC with fluorescence detection.

2. Experimental

2.1. Materials and reagents

Amprolium hydrochloride was purchased from Sigma (St. Louis, MO, USA). Beclotiamine, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl-5-(2-chloroethyl)-4-methylthiazolium chloride, as an internal standard (I.S.), was kindly supplied from Sankyo (Tokyo, Japan). All other reagents were of analytical-grade.

2.2. Chromatography

The HPLC system consisted of two Work-21 MP-312 pumps, a Gasorr GT-104 de-airation apparatus (both Labosystem, Tokyo, Japan), an AS-950 intelligent sampler, an 860-CO column oven, an 821-FP intelligent spectrofluorometer-detector (Jasco, Tokyo, Japan), a signal cleaner SC77 (System instruments, Tokyo, Japan) and a CR8A chromatopak detector (Shimadzu, Kyoto, Japan). The analytical column used was a Capcell pack C_{18} column (UG-120, 5 μm, 250×4.6 mm I.D., Shiseido, Tokyo, Japan) protected by Sumipax PG-ODS-filter (Sumika Chemical Analysis Service, Ohsaka, Japan). HPLC analysis was performed at 40°C with a mobile phase of 0.2 *M* potassium dihydrogenphosphate–acetonitrile (9:1, v/v) containing 5 m*M* 1-hexanesulfonic acid sodium salt. A reaction coil (10 m×0.25 mm I.D.) made from stainless steel tubing was maintained at 40°C. The post-column reagent was prepared by dissolving 50 g of sodium hydroxide and 0.8 g of potassium ferricyanide in 1 l of distilled water. This reagent was confirmed as stable for 24 h at room temperature if stored in the dark. The flow-rates of the mobile phase and the post-column reagent were 0.6 ml/min. The mobile phase was filtered through a 0.45 μm membrane filter prior to use. An acetonitrile–water (1:9, v/v) solution was devoted to washing the injection unit. The fluorescence detector was set at 400 and 460 nm for excitation and emission, respectively. The ratio of the peak area of APL to that of I.S. was used as the assay parameter.

2.3. Calibration

Stock solutions of APL and I.S. were prepared using 0.33 *M* perchloric acid to yield a final concentration 100 ng/ml. These solutions were stored at 4°C for 1 month and used within 1 month. The calibration curves for APL were obtained by spiking blank plasma with the stock solution, to yield 5, 10, 25, 50, 80, 100 ng/ml of APL.

2.4. In vivo study

Two white-leghorn hens (1.38 and 1.41 kg, 17 weeks old) were used. The chickens were given a SD food (Nisseikenn, Tokyo, Japan), containing no drug, and water ad libitum, and acclimatized for 3 months. The chickens were fasted for 24 h prior to, and for 13 h after, oral administration of APL (13 or 26 mg/kg) as solution. Blood samples were taken in heparinized tubes from the sub-wing vein at 0.5, 0.75, 1, 1.5, 2, 3, 5, 7, 13, 24, 31 and 48 h after drug administration. Blood samples were centrifuged and the collected plasma was stored at −20°C until analysis.

2.5. Sample preparation

A 0.2-ml volume of plasma was placed in test tube and mixed for 30 s using vortex mixer after addition of 0.1 ml of I. S. solution and 0.5 ml of 0.33 *M* perchloric acid solution. After 10 min of centrifugation at 2150 *g*, the supernatant was separated and allowed to stand over 3 h. A 30-μl aliquot of the supernatant was injected into HPLC system.

3. Results and discussion

Some HPLC methods for determination of APL in muscle [5,6] or in liver [7] have been reported. Yamaguchi et al. used 1-[(4-amino-2-methyl-5-pyrimidynyl)methyl]-pyridinium chloride hydrochloride (PB1) as I.S. [6] for HPLC determination of APL in muscle. However, we could not use that I.S. because we found a peak at almost the same position

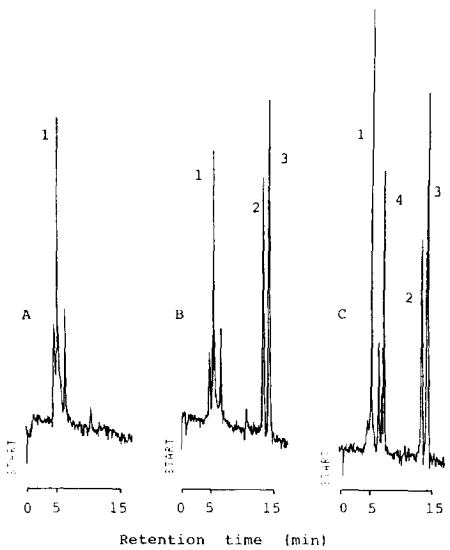

Fig. 1. Chromatograms of (A) blank plasma, (B) blank plasma spiked with amprolium (50 ng) and beclotiamine (10 ng) and (C) plasma sample after oral administration of 26 mg/kg amprolium to chicken. Peaks: 1=thiamine, 2=beclotiamine, 3=amprolium, 4=unknown metabolite of amprolium.

as that of PB1 on the chromatogram from the plasma sample after APL administration to chicken.

We tried to use the determination method for thiamine which has a similar structure as APL in plasma. This method is based on pre-column HPLC with fluorescence detection [8,9]; however, we could not obtain good results with this method, because the detected area ratio of APL increased with time after sample preparation.

In order to develop a rapid, simple and sensitive method for APL in plasma, we used only one step for sample preparation and detected the post-column derivative using fluorometric detection. In addition, the sample size required for analysis was reduced to 0.2 ml of plasma. In spite of this small volume of plasma, the limit of detection (i.e., the lowest concentration yielding a signal consistently three times above the baseline noise) was 2 ng/ml and the limit of quantitation (the lowest concentration that can be measured with acceptable accuracy and precision) was 5 ng/ml.

Typical chromatograms of drug-free plasma, plasma spiked with APL and I.S. and plasma taken after oral administration of APL are shown in Fig. 1. The chromatogram of drug free plasma showed no interfering peaks at the retention time of APL and I.S. The retention times of APL and I.S. were 14 and 13 min, respectively. The peaks of incidental thiamine and the derivative showed faster retention times than APL and I.S. Ethopabate, a co-administered drug, was not detected with fluorometric detection. An unknown peak, which was thought to be a metabolite of APL, was detected only in samples analysed after APL administration. This peak appeared at almost the same position as PB1 that was used as I.S. in the report by Yamaguchi et al. [6]. Therefore, we had to find a new internal standard, beclotiamine, for the determination of APL using HPLC.

The extraction recoveries of APL from plasma containing 20, 50 and 100 ng/ml APL were 98, 99 and 105%, respectively. The results of the accuracy and precision (intra-day and inter-day) of the method are given in Table 1, and are all below 15%, which is an acceptable range for validated HPLC methods. The accuracy and repeatability were improved by allowing the supernatant to stand for 3 h. The calibration curves were linear in the 5–100 ng/ml range of the assay and a good correlation coefficient (r) was consistently found over five different days [$r=0.9991\pm0.00092$ (mean±S.D.)]. The slope of the calibration curve was 0.03004 ± 0.00305 (mean±S.D., $n=5$). The inter-day variability in the slope was 10.1%.

Table 1
Accuracy, intra-day and inter-day precision and recovery for the analysis of amprolium in plasma

Theoretical concentration (ng/ml)	Accuracy (mean±C.V.) (%)	R.E. (%)	Precision (%)		Recovery (mean±C.V.) (%)
			Intra-day	Inter-day	
20	96.55±4.43	−3.45	5.82	11.18	97.70±10.7
50	94.59±7.48	−5.41	4.28	5.32	98.61±4.1
100	94.75±5.84	−5.25	7.75	7.21	105.09±5.3

$n=6$.

Spiked plasma samples of APL were stored at −20°C and no significant degradation of APL was found during storage for a 3-month period. The plasma concentration–time profile after oral administration of APL to chicken is shown in Fig. 2.

In conclusion, the HPLC method described in this report is simple, sensitive, reproducible and able to treat numerous samples in a short period of time. Furthermore, the assay is applicable to pharmacokinetic studies of APL in chicken.

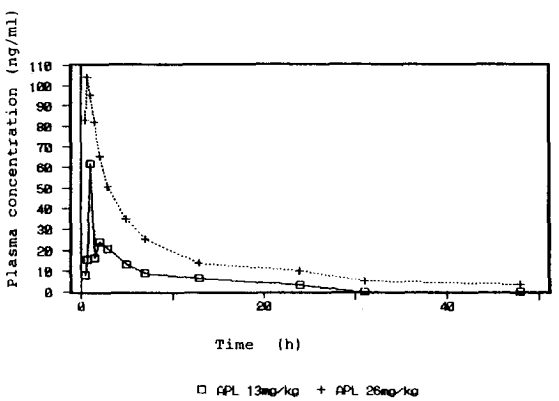

Fig. 2. Plasma concentration–time curve of amprolium after oral administration of 13 or 26 mg/kg amprolium to a chicken.

Acknowledgments

The authors are very grateful to Sankyo for cordial supply of beclotiamine.

References

[1] B. Lang, Fleiscwirtschaft, 69 (1989) 524.
[2] D. Polin, E.R. Wynosky and N.J. Porter, Proc. Soc. Exp. Biol. Med., 114 (1963) 273.
[3] A. Chahar, J.S. Yadav, S.N. Sharma and U.K. Vyas, Ind. Vet. J., 70 (1993) 411.
[4] F.M. Loew and R.H. Dunlop, Am. J. Vet. Res., 33 (1972) 2195.
[5] T. Nagata and M. Saeki, J. Assoc. Off. Anal. Chem. Int., 69 (1986) 941.
[6] T. Yamaguchi, J. Hanai, M. Hirata, Y. Shiroisi, M. Aoki and N. Takasugi, J. Food Hyg. Soc. Jpn., 25 (1984) 499.
[7] K.D. Gallicano, H. Park, J. Yee, L.M. Young and P.W. Saschenbrecker, J. Assoc. Off. Anal. Chem. Int., 71 (1988) 48.
[8] J. Bontemps, P. Philippe, L. Bettendorff, J. Lombet, G. Oandrifosse, E. Schoffeniels and J. Crommen, J. Chromatogr., 307 (1984) 283.
[9] L. Bettendorff, C. Grandfils, C. de Rycker and E. Schoffeniels, J. Chromatogr., 382 (1986) 297.