ELSEVIER

Journal of Chromatography B, 696 (1997) 166–171

JOURNAL OF CHROMATOGRAPHY B

Short communication

Immunoaffinity column cleanup procedure for analysis of ivermectin in swine liver

Jun Suo Li[a,*], Xi Wang Li[b], Hong Bo Hu[a]

[a]*Department of Veterinary Pharmacology, Pharmacy and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100094, People's Republic of China*

[b]*Department of Veterinary Medicine, Zhangjiakou Agricultural College, Hebei 075100, People's Republic of China*

Received 11 February 1997; revised 21 March 1997; accepted 1 April 1997

Abstract

A simple and sensitive method for the analysis of ivermectin (22,23-dihydroavermectin B_1) in swine liver based on immunoaffinity column cleanup is described. The immunosorbent was prepared by coupling polyclonal anti-ivermectin antibodies to carbonyl diimidazole-activated Sepharose CL-4B. After extraction with methanol, ivermectin was cleaned up on an immunoaffinity column, and determined by reversed-phase liquid chromatography with UV absorbance detection at 245 nm. Recoveries of ivermectin from fortified samples of 5–100 $\mu g\,kg^{-1}$ levels ranged 85–102%, with coefficients of variation of 6–12%. The limit of detection was 2 $\mu g\,kg^{-1}$ in a 5-g sample. © 1997 Elsevier Science B.V.

Keywords: Ivermectin

1. Introduction

Ivermectin (22,23-dihydroavermectin B_1), derived from a naturally-occurring fermentation product, is an important antiparasitic agent with very potent broad spectrum (Fig. 1) [1]. It consists of 2 homologues: not less than 80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and not more than 20% 22,23-dihyroavermectin B_{1b} (H_2B_{1b}). Ivermectin is used in a wide variety of hosts, with a dosage typically 0.2 $\mu g\,kg^{-1}$ body weight [2]. Liver is the target tissue for residue control, and the maxium residue limit (MRL) for H_2B_{1a} (a marker residue) is 15 $\mu g\,kg^{-1}$ [3]. Tolan et al. [4] and Tway et al. [5] were the first

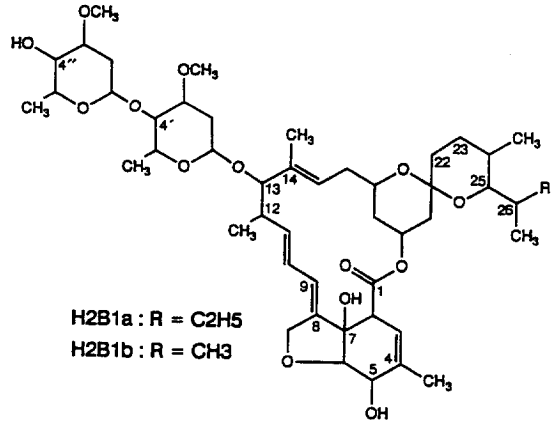

Fig. 1. The structure of ivermectin.

*Corresponding author.

0378-4347/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0378-4347(97)00220-X

to describe a liquid chromatographic (LC) method with fluorescence detection, based on which many analytical procedures have been reported [6–10]. Currently, the methods are sensitive (with limits of detection of 1–3 $\mu g\,kg^{-1}$) and specific enough to analyze ivermectin residue in animal tissues, but they involve tedious cleanup and fluorescence derivatization steps. Reuvers et al. [11] developed a relatively rapid direct LC method with UV absorbance detection.

Recently, we used immunoaffinity columns, prepared by coupling the antibodies to a cyanogen bromide (CNBr)-activated support, to analyze avermectin B_1 residue in cattle tissues [12], and ivermectin in sheep serum [13]. An enzyme-linked immunosorbent assay (ELISA) was also developed to determine avermectin B_1 in cattle tissues [14]. This paper describes a simple one-step cleanup procedure for ivermectin in swine liver, using an immunoaffinity column prepared by an alternative activation/coupling procedure with carbonyl diimidazole (CDI).

2. Experimental

2.1. Reagents

Ivermectin stock solution (100 $\mu g\,ml^{-1}$ of H_2B_{1a} and 7.6 $\mu g\,ml^{-1}$ of H_2B_{1b}) was provided by BAU Newtech Development (Beijing, PRC), and stored at −20°C. Ivermectin working solutions were prepared by diluting the stock solution with mobile phase.

Water was redistilled with all-glass apparatus. Ethyl acetate (99%) obtained from Beijing Chemical (Beiijng, PRC) was redistilled with all-glass apparatus. Methanol and acetonitrile were LC grade from Beijing Chemical. Sepharose CL-4B (46–165 μm) was obtained from Pharmacia (Uppsala, Sweden), CDI (97%) from Fluka (Buchs, Switzerland). All other reagents were analytical grade or better.

Phosphate-buffered solution (PBS) was prepared by dissolving 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KCl and 8.8 g NaCl in 900 ml of water. This solution was adjusted to pH 7.4 with 2.0 $mol\,l^{-1}$ NaOH, and diluted to 1 l with water. PBS containing 0.5 $mol\,l^{-1}$ NaCl was prepared in the same way as above except 29.3 g of NaCl was used. The coupling buffer solution was 0.13 $mol\,l^{-1}$ boric acid (pH 8.5). The blocking solution was 1.0 $mol\,l^{-1}$ ethanolamine (pH 8.0). The acetate buffer contained 0.1 $mol\,l^{-1}$ sodium acetate and 0.5 $mol\,l^{-1}$ sodium chloride (pH 4.0).

2.2. Apparatus

The instruments used were a homogenizer, Model AM-6 (Nihonseiki Kaisha, Tokyo, Japan), a Vortex mixer, Model WH861 (Taicang Biochemical Instrument, Jiansu, PRC), a magnetic stirrer, Model 79HW-1 (Recheng Electrical Equipment, Zhengjiang, PRC), a rotary evaporator, Model XZ-6 (Kelong Instrument, Beijing, PRC), a shaking apparatus, Model SHZ-82 (Taicang Biochemical Instrument), a centrifuge, Model LD4-2A (Beijing Medical Equipment, Beijing, PRC), a UV–Vis spectrophotometer, Model 751GW (Shanghai Analytical Instrument, Shanghai, PRC), a glass column for packing immunosorbent, 10×0.17 cm I.D., with a fritted disc (porosity 40–60 μm) sealed into the bottom and a 50-ml reservoir connected to the top with ground-glass joints (Beijing Chemical).

The LC system was composed of a SP8810 pump, a SP8450 UV–Vis detector, and a SP4270 integrator (Spectra-Physics, San Jose, CA, USA). The analytical column was a Brownlee column packed with 5-μm C_{18}, 220×4.6 mm I.D. (Applied Biosystems, San Jose, CA, USA), and used at room temperature. The mobile phase was acetonitrile–methanol–water (9:9:2, v/v) at a flow-rate of 1.0 $ml\,min^{-1}$. The detection wavelength was 245 nm (AUFS=0.02). The injection volume was 100 μl.

2.3. Antibodies preparation

The polyclonal anti-ivermectin antibodies were obtained by immunizing New Zealand rabbits with 4″-*O*-hemisuccinoylavermectin B_1-bovine serum albumin [15]. The antibodies can recognize avermectin B_1 and ivermectin specifically. The immunoglobulin G (IgG) fraction of the antisera was purified by ammonium sulfate precipitation and diethylaminoethyl cellulose anion-exchange chromatography [12],

and stored at −20°C. Just before preparation of the immunosorbent, IgG was diluted to 5 mg ml^{-1} with coupling buffer solution.

2.4. Immunosorbent preparation

The procedures used for solvent exchange of the matrix from water, activation and coupling were based on the protocol of Bethel et al. [16]. A 5-ml volume of Sepharose CL-4B was washed over a sintered-glass funnel by suction, sequentially with 20 ml of water, 20 ml of acetone–water (1:1, v/v) and four 10-ml volumes of acetone. The moist cake was suspended in 6 ml of acetone in a beaker, and 0.2 g CDI was added. The mixture was agitated slowly with a magnetic stirrer for 20 min at room temperature. The suspension was filtered, and washed with 50 ml of acetone and four 10-ml aliquots of coupling buffer solution. This cake was added to 5 ml of coupling buffer solution containing 25 mg of IgG, and agitated slowly for 24 h at 4°C. After centrifugation for 5 min at 300 *g*, the supernatant was collected to determine coupling efficiency with a UV–Vis spectrophotometer. The residual active sites were blocked by suspending the gel in 6 ml of 1.0 mol l^{-1} ethanolamine and agitating slowly for 4 h at room temperature. After washing with PBS, the immunosorbent was stored in PBS–0.02% sodium azide at 4°C.

2.5. Column capacity determination

A relatively large amount of ivermectin (4000 ng H_2B_{1a} in 50 ml of PBS–methanol, 85:15, v/v) was drawn through an immunoaffinity column of 1.0-ml bed volume continuously at a flow-rate of 1.2 ml min^{-1} by gentle suction. The ivermectin-saturated column was washed with 40 ml of PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) and 10 ml of water–methanol (8:2, v/v). Ivermectin was eluted with 3 ml of 100% methanol, and determined by LC with UV absorbance detection at 245 nm. The column was regenerated by washing with 5 ml of water and 10 ml of PBS, and stored in PBS–0.02% sodium azide at 4°C. The column capacity was determined periodically during use and storage.

2.6. Sample preparation/immunoaffinity column cleanup

Partially thawed swine liver was minced, and homogenized with a homogenizer for 2 min at high speed. The homogenate was stored at −20°C in sealed plastic bags.

A 5.0-g thoroughly thawed homogenate was transferred to a 50-ml graduated polypropylene centrifuge tube, and 15.0 ml of methanol was added. The mixture was shaken thoroughly by hand and again by using a shaking apparatus for 1 h at medium speed. The sample was adjusted to a volume of 20 ml with a little methanol and shaken thoroughly. After centrifugation for 5 min at 2000 *g*, 10 ml of supernatant was collected and mixed with 40 ml of PBS. This solution was subjected to immunoaffinity column cleanup procedure.

The steps for adsorption, washing and elution were the same as described in column capacity determination. After evaporation to less than 1 ml with a rotary evaporator at 55°C, the eluate was extracted with 5 ml ethyl acetate using a Vortex mixer for 15 s. The organic layer was collected and evaporated to dryness at 55°C. The residue was redissolved in 1 ml mobile phase with a Vortex mixer for 15 s. After filtration through a 0.45-μm disposable filter, aliquots of 100 μl were used for LC analysis.

2.7. Calibration curve and fortification

The standard calibration curve for H_2B_{1a} covered a concentration range of 10–500 ng ml^{-1}. Blank swine liver homogenates were fortified with 10–50 μl of ivermectin standard solution at 5–100 μg kg^{-1} levels and mixed thoroughly. After 10–15 min, the samples were extracted, cleaned up and determined as described earlier. The levels of ivermectin (H_2B_{1a}) in the sample were calculated with the following equation:

$$\text{ivermectin, } \mu g\ kg^{-1} = (2 \times C \times V)/W$$

here C (ng ml^{-1}) is the concentration of ivermectin in the final sample solution, determined from the standard curve, V (ml) is the volume of the final

sample solution, and W (g) is the weight of the sample.

3. Results and discussion

3.1. Immunoaffinity columns

The advantages of the CDI method for preparation of immunosorbent over the classical CNBr method is the absence of charged groups introduction in the former, the ease of handing of the reagent and the stability of the product [16]. Compared with the CNBr method, the reaction procedure of the CDI method is simple, with only *N*-substituted carbamates produced, and has higher activation efficiency and matched-coupling efficiency. In this experiment, the coupling efficency of IgG to CDI-activated Sepharose CL-4B was nearly 100%, which resulted in the immunosorbent with IgG loading of 5 mg ml^{-1} gel. The dynamic column capacity was 2546 ng H_2B_{1a} per ml of gel, and the specific column capacity was 509 ng H_2B_{1a} per mg of immobilized IgG, which are slightly higher than those of the earlier CNBr method [13].

The total column capacity of 1-ml bed volume tends to decrease in a way similar to previous reports [12,13]: 54% (1375 ng of H_2B_{1a}) remained after 16 cycles of reuse during one month. There was no significant change in capacity during storage of at least one year in PBS–0.02% sodium azide at 4°C.

3.2. Extraction and cleanup

Methanol was used for extraction, not only because it can extract ivermectin from liver quantitatively and precipitate proteins well, but also since this extract can easily be subjected to immunoaffinity column cleanup. Ivermectin has extremely low water-solubility (only 4 mg l^{-1} at room temperature). Therefore, PBS (or water)–methanol was used throughout the cleanup procedure in order to increase solubility of ivermectin in aqueous media and reduce adsorption of ivermectin on glassware. Pre-wetting glassware (especially, the reservoirs of immunoaffinity columns) with a few millilitres of methanol can improve the recovery of ivermectin. Methanol is usually used for eluting haptens from immunosorbent, and ivermectin can be completely eluted from an ivermectin-saturated column of 1-ml bed volume with only 3 ml of methanol. Thus, only one organic solvent was used in the preparation of the sample, which simplified the procedure.

The earlier-reported LC method based on a cleanup procedure with immunoaffinity columns prepared by the CNBr method [12,13] did not work well in the analysis of ivermectin in swine liver (a more complicated sample). Ivermectin cannot be resolved and detected by the LC method because of matrix interference from nonspecific adsorption of the immunosorbent. Certain improvement was obtained using immunoaffinity columns prepared by the CDI method and alternative LC conditions described in the present paper. Furthermore, an ivermectin-adsorbed column was washed sequentially with 40 ml of PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) and 10 ml of water–methanol (8:2, v/v) in order to reduce nonspecific adsorption of the interfering matrix, by disrupting the ion-exchange, hydrophobic reaction or hydrogen bond, possibly occurring between the sample matrix and the immunosorbent. In the adsorption step, there was ca. 15% (v/v) methanol in sample solutions, which was higher than that of earlier reports [12,13]. This washing or adsorption step should not affect the antibody–ivermectin complex, for the immune reacion between the antibodies and ivermectin can tolerate up to 1.5 mol l^{-1} NaCl or 20% (v/v) of methanol [14]. Another reason for washing columns with water–methanol (8:2, v/v) before elution with methanol was to avoid crystallization of salts in PBS that would clog the column. Fig. 2 shows the LC chromatogram of ivermectin, blank swine liver and fortified sample. No interference of the matrix was detected. Moreover, an ethyl acetate extraction step after elution was not always necessary, depending on the water content of the eluate.

3.3. Determination and fortification

The standard calibration curve for H_2B_{1a} (concentration versus peak height) was linear ($r^2 = 0.9999$, $n = 6$) in the concentration range of 10–500 ng ml^{-1}.

The results of fortification studies are shown in Table 1. Recoveries of ivermectin were 85–102% in

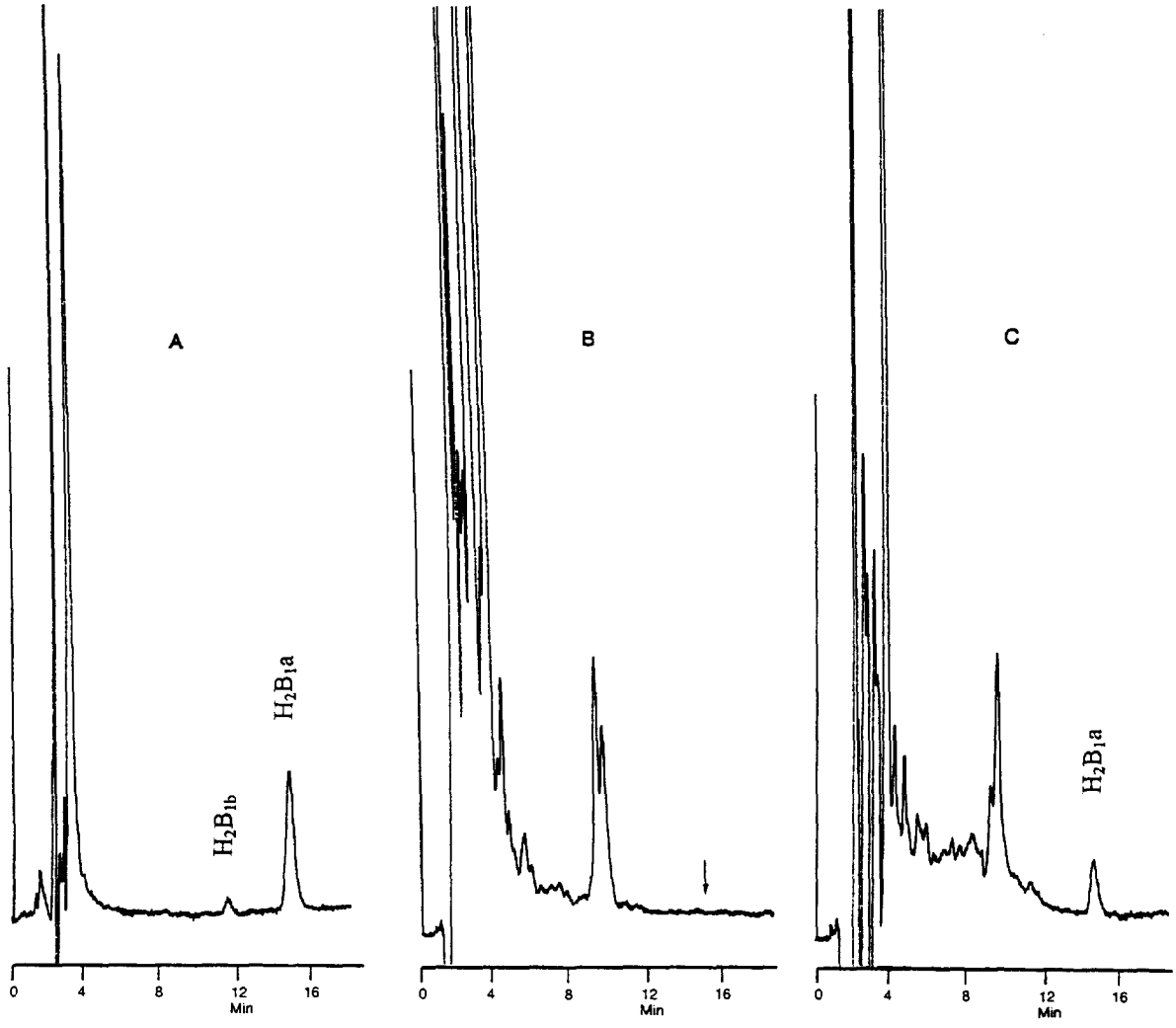

Fig. 2. Chromatogram of swine liver after extraction with methanol, immunoaffinity cleanup and LC analysis. The LC conditions are described in Section 2.2. (A) 100 ng ml^{-1} of ivermectin (H_2B_{1a}) in mobile phase, (B) blank swine liver, and (C) swine liver fortified with 20 μg kg^{-1} of ivermectin.

Table 1
Recoveries of ivermectin (H_2B_{1a}) from fortified swine liver

Added (μg kg^{-1})	Determined (μg kg^{-1})[a]	Recovery (%)	C.V. (%)
0	–	–	–
5	4.3±0.3	86	7
20	17.0±1.0	85	6
50	44.1±3.2	88	7
100	102.2±12.5	102	12

[a] Values are mean±standard derivation, $n=4$.

fortified levels of 5–100 $\mu g\,kg^{-1}$, with coefficients of variation (C.V.s) of 6–12%. The limit of detection, defined as the lowest concentration that can be determined to be statistically different from a blank, was 2 $\mu g\,kg^{-1}$ [17]. The limit of determination was 5 $\mu g\,kg^{-1}$ in this study. High recovery and sensitivity were due to the simplicity and specificity of the immunoaffinity column cleanup procedure. The above analytical results, obtained by the LC method with direct UV absorbance detection, are comparable to those of a fluorescence derivatization LC method. This is one of the simplest methods for determing ivermectin residue in animal tissues yet reported, with only one chromatographic separation step involved in the cleanup procedure. It is sensitive and reliable enough for determining ivermectin residue in liver tissue.

This work demonstrates the high specificity of antibody-mediated cleanup (AMC) procedures, as do the previous reports of Van de Water and Haagsma [18] and van Ginkel [19]. It is certain that AMC, such as immunoaffinity column cleanup, can simplify preparation of samples and improve analytical quality. However, it is also clear that AMC cannot always or completely eliminate matrix interference of samples. Perhaps nonspecific adsorption is a crucial problem encountered by every researcher and user. In analysis of pesticide/drug residues in biological samples, even small nonspecific adsorption would have significant or deleterious effects on analytical performances, such as the limit of detection and selectivity. Suitable activation/coupling and washing steps are the key to a well-designed immunoaffinity column cleanup procedure. In some case, other cleanup steps before or after this procedure are necessary.

Acknowledgments

This work is partly financed by the China Natural Science Foundation (No.39600112).

References

[1] W.C. Campbell, M.H. Fisher, E.D. Stapley, G. Albers-Schonberg, T.A. Jacob, Science 221 (1983) 823.
[2] W.C. Campbell, G.W. Benz, J. Pharmacol. Ther. 7 (1994) 1.
[3] FAO Food and Nutrition Paper 41/3, Residues of Some Veterinary Drugs in Animal Tissues, the Thirty-sixth Meeting of the Joint FAO/WHO, Expert Committee on Food Additives, Rome, February, 1990, p. 45.
[4] J.W. Tolan, P. Escola, D.W. Fink, H. Mrozik, L.A. Zimmerman, J. Chromatogr. 190 (1980) 367.
[5] P.C. Tway, J.S. Wood, G.V. Dowing, J. Agric. Food Chem. 29 (1981) 1059.
[6] P. De Montigny, J.S.K. Shim, J.V. Pivninchny, J. Pharm. Biomed. Anal. 8 (1990) 507.
[7] S.V. Prabhu, T.A. Wehner, P.C. Tway, J. Agric. Food Chem. 39 (1991) 1468.
[8] F.J. Schenck, S.A. Barker, A.R. Long, J. AOAC Int. 75 (1992) 655.
[9] C.D.C. Salisburg, J. AOAC Int. 76 (1993) 1149.
[10] J.M. Degroot, B. Wyhowski de Bukanski, S. Srebrnik, J. Liq. Chromatogr. 17 (1994) 1419.
[11] R.Th. Reuvers, R. Diaz, M. Martin de Pozuelo, M. Ramos, Anal. Chim. Acta. 275 (1993) 353.
[12] J.S. Li, C.F. Qian, J. AOAC Int. 79 (1996) 1062.
[13] J.S. Li, S.Q. Zhang, J. AOAC Int. 79 (1996) 1300.
[14] J.S. Li, C.F. Qian, Acta. Vet. Zootech. Sin. 28 (1997) 77.
[15] J.S. Li, C.F. Qian, Acta. Vet. Zootech. Sin. 27 (1996) 531.
[16] G.S. Bethell, J.S. Ayes, M.T.W. Hearn, W.S. Hancock, J. Chromatogr. 219 (1981) 353.
[17] S.G. Gorbach, U. Bos, H.-P. Thier, H. Frehse, W.D. Weinmann, in: H.-P. Thier, H. Zeumer (Eds.), Manual of Pesticide Residue Analysis, vol. 1, VCH, Weinheim, 1987, p. 37.
[18] C. van de Water, N. Haagsma, J. Chromatogr. 411 (1987) 415.
[19] L.A. van Ginkel, J. Chromatogr. 561 (1991) 363.