Distribution of Total ^{14}C Residue in Egg Yolk, Albumen, and Tissues Following Oral [^{14}C]Sulfamethazine Administration to Hens

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The distribution of total ^{14}C residues was studied in egg yolk and albumen after administration of either single or multiple oral dosages of [^{14}C]sulfamethazine (SMZ). One day after a single dose of [^{14}C]SMZ (121 mg of sulfamethazine, 2.42×10^7 dpm), the ^{14}C residue concentration peaked in egg albumen and egg yolk with the concentration in the former >4-fold greater than in the latter. Three days postdose, the ^{14}C residue concentration in the yolk was ~7-fold higher than in the egg albumen. A multiple dose of [^{14}C]SMZ containing sulfamethazine mass equivalent of an average therapeutic dose (282 mg, 2.9×10^7 dpm) for chickens was also administered orally for six consecutive days to hens. A significantly reduced level of egg production was observed during the medication, and most of the hens stopped laying eggs after the last dose. The ^{14}C residue concentrations peaked on the last day (sixth) of medication in egg albumen and yolk. The ^{14}C residue concentrations were also measured in liver, muscle, blood, and plasma of chickens sacrificed at 1, 24, 48, and 72 h after the last dose. Highest concentrations of ^{14}C residue were accumulated in liver followed by, in decreasing order, blood, plasma, and muscle.

Keywords: *Sulfa drugs; sulfamethazine; total residue; eggs*

INTRODUCTION

Sulfonamide drugs are widely used in poultry for the clinical treatment of bacterial diseases and for incorporation into feed at the subtherapeutic level to promote growth. Sulfamethazine (SMZ) is approved for use in broiler chicks for the prevention and treatment of various bacterial diseases (*Code of Federal Regulations*, 1997). It is, however, not approved for use in laying hens. Because of the widespread use and availability of SMZ, its unapproved use in poultry and other species is a possibility and may result in violative residues in eggs and other edible products. The residue may include parent drug as well as metabolites. Therefore, information about the disposition of SMZ and its metabolite in eggs is needed. A few studies in which hens were orally dosed with non-radiolabeled drugs have been reported to determine the depletion and metabolism of SMZ in eggs. In both single- and multiple-dose oral studies (gelatin capsules), the concentration of SMZ in egg albumen peaked at 24 h and exceeded that in the yolk after the last treatment (Geertsma et al., 1987). Trace levels of N_4-acetyl and 6-methyl hydroxy metabolites of SMZ were also detected. When the dose was given in drinking water for 5 days, the concentrations in both egg albumen and yolk were about the same after 1 day of withdrawal, and the presence of metabolite was not detected (Roudaut, 1993). In a recent study (Shaikh et al., 1999), using a single oral dose (gelatin capsule), SMZ concentrations in albumen peaked at 48 h and exceeded those in egg yolk after the last dose. In addition, a small concentration of the N_4-acetyl metabolite was also detected in both albumen and yolk. The advantage of using ^{14}C-labeled sulfa drugs has been demonstrated in a number of studies, for example, the metabolism of [^{14}C]SMZ in swine (Paulson et al., 1981) and [^{14}C]-sulfadimethoxine in lobster (Barron and James, 1994) and swine (Adams et al., 1996). Therefore, this study was initiated to determine the distribution of [^{14}C]SMZ in egg albumen, yolk, and tissues.

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MATERIALS AND METHODS

Apparatus. The liquid chromatographic (LC) system consisted of a Hewlett-Packard (HP) model 1050 system (Palo Alto, CA) fitted with a quaternary pump, an autosampler (series 1100), a column heater, a solvent degasser, a multichannel interface (35900), HP ChemStation software (DOS series), an HP Laser Jet 6P printer, a *β-RAM* radioactivity flow-through detector for HPLC (IN/US Systems, Inc., Tampa, FL), and a Dell Optiplex GX computer.

The analytical (150 mm × 4.6 mm) and guard (7.5 × 4.6 mm) columns employed were LiChrosorb RP-8 (Alltech Associates, Deerfield, IL), with a packing of 5 μm particles. Both the analytical and guard columns were in a column heater set at 35 °C.

All centrifugations were carried out at 4100 RCF using a swing-out rotor (M4) in a Jouan CR 422 refrigerated centrifuge (Jouan Inc., Winchester, VA) set at 4 °C for 15 min. Polypropylene tubes, Falcon round (14 mL), and conical bottoms (15 mL) were used (Corning Glass Works, Corning, NY). All liquid transfers were made with Eppendorf digital pipets.

Combustion of egg yolk samples was carried out in a Packard model 301 sample oxidizer (Packard Instruments, Meriden, CT). Egg albumen samples and effluents from the oxidizer after combustion of egg yolk samples were counted in a model 3801 Beckman liquid scintillation counter (Beckman Instruments Co., Fullerton, CA) using Packard Insta-gel XF as liquid scintillation counting fluid.

Chemicals and Reagents. Glass-distilled organic solvents (Burdick & Jackson Laboratories, Muskegon, MI) and water from a Milli-Q plus ultrapure water system (Millipore Corp.,

10.1021/jf000519e
Published on Web 12/01/2000

Bedford, MA) were used. All chemicals were of HPLC grade, except where noted. [^{14}C]SMZ and a [^{14}C]sulmet mixture containing SMZ, N_4-acetyl-SMZ, desamino-SMZ, and glucosyl-SMZ were gifts from Dr. Gaylord Paulson of ARS/USDA, Metabolism and Radiation Research Laboratories, Fargo, ND. A Tru-Count liquid scintillation cocktail for an on-line HPLC radioactivity detector was obtained from IN/US, Inc.

HPLC Mobile Phase. A stock solution of 0.5 M potassium phosphate was prepared by weighing 68 g and transferring with water to a 1-L glass volumetric flask; additional water was added to the mark and mixed. The mixture was refrigerated until used. A mobile phase buffer, 0.01 M KH_2PO_4, was prepared by transferring 20 mL of 0.5 M stock buffer solution into a 1-L flask; additional water was added to reach the mark and mixed. No pH adjustment was made. The gradient mobile phase was used and consisted of an initial combination of 5% acetonitrile in 0.01 M phosphate buffer solution for 5 min, gradient to 15% acetonitrile in 15 min, holding for 20 min, and gradient returned to 5% acetonitrile in 35 min. A postequilibration time of 5 min was used before the next injection.

Preparation of Standard Solutions. Eleven microcuries of [^{14}C]SMZ (specific activity = 5.06 mCi/mmol) received in a glass scintillation vial was dissolved in 10 mL of methanol to give a primary stock solution of 1.1 μCi/mL. The secondary stock solution of 0.11 μCi/mL was prepared by transferring 1 mL of the primary stock solution of [^{14}C]SMZ into another scintillation vial and by adding 9 mL of methanol. The working standard solution was prepared by transferring 100 μL of secondary stock solution into a 15-mL polypropylene centrifuge tube and adding 900 μL of a mobile phase solution (15% ACN/buffer). Other dilutions were made as appropriate. The primary stock solution was stored at −20 °C, and other solutions were refrigerated until used. The standard mixture containing [^{14}C]SMZ and its ^{14}C metabolites, glucosyl-SMZ, N_4-acetyl, and desamino-SMZ, were dissolved in 1 mL of methanol. The average specific activity for each was 1054 dpm/μg.

Standard Curves. For radiochromatogram analysis, the standard curves using various volumes of secondary and working standard solutions were prepared and found to be linear. The disintegrations per minute (dpm) values from 7.5, 15, 30, and 45 μL of working standard solution injected were plotted against their peak heights, and a correlation coefficient >0.98 was obtained. Similarly, the dpm values from 5, 7.5, 10, and 20 μL injections of secondary standard solution injected were plotted against their peak heights and gave a correlation coefficient of >0.99. The limit of quantitation (LOQ) was 183 dpm (4.5 ng), which is the lower end (7.5 μL) of the standard curve, and the limit of detection (LOD) was 122 dpm (3.0 ng). To determine the reproducibility of response, 10 μL of secondary standard solution was injected in triplicates. The average CV values for peak height and peak area were 9 and 12%, respectively, and peak heights were used throughout the calculations.

Animal Experiments. White Leghorn hens were used, and control eggs were obtained prior to dosing. Initially in a pilot study, a single dose of 121.4 mg of [^{14}C]SMZ (specific activity = 200 dpm/μg) was orally administered in a gelatin capsule to a hen. Eggs were collected at daily intervals of 24 h until 9 days after the treatment. This was followed by a multidose study in which 10 chickens were orally dosed for six consecutive days with an average dose of 274.6 mg/day of [^{14}C]SMZ (specific activity = 100.5 dpm/μg). This dose approximates the approved dose for broiler chickens (*Code of Federal Regulations*, 1997). Eggs were scheduled to be collected at 24 h intervals during dosing and for 10 days after the last dose. However, most of the hens laid eggs infrequently during dosing, and all except one hen stopped laying eggs after the last dose. Egg albumen and egg yolk were separated and transferred into polypropylene tubes and stored at −20 °C, until assayed.

Four chickens were also sacrificed: one each at 1 h, 1 day, 2 days, and 3 days after the last dose. Blood, liver, and skeletal muscle were collected at sacrifice from each chicken and stored at −20 °C.

Sample Preparation for Combustion Analysis. Triplicate samples of ~0.5 g each of yolk, liver, muscle, and blood were weighed into a combustocone containing an absorbent pad and transferred into an ignition basket of the Packard sample oxidizer. The sample oxidizer consists of combustion and ^{14}C collection systems, where a sample is combusted in an oxygen atmosphere. During combustion, radioactive carbon dioxide is formed as the oxidized form of ^{14}C. The $^{14}CO_2$ is trapped in a column filled with carbon dioxide absorbent (Packard Carbo-Sorb E) and forms a carbamate, which is flushed into a vial with a scintillator (Packard Permafluor E$^+$) for ^{14}C. The sample vial is then placed into a Beckman Scintillation counter to measure radioactivity for 20 min.

For recovery purposes, five samples each of control tissue fortified with 50 μL of 0.011 μCi/mL (1121 dpm), control tissue, and [^{14}C]SMZ standards (1121 dpm) were combusted as above. The control sample counts were subtracted from the fortified sample counts. The average recoveries of ^{14}C from the combustion of [^{14}C]SMZ fortified egg yolk, liver, muscle, and blood were determined to be 96, 100, 93, and 89%, respectively, with coefficient of variations (CV) of 4, 1, 2, and 0.4%, respectively. The sample oxidizer has been frequently used for combustion of solid samples, for example, tissue and feces (Adams et al., 1996; Paulson et al., 1981, 1985) to measure radioactivity.

Direct Counting of Albumen and Plasma Samples. Albumen or plasma samples (~0.5 g) were miscible with liquid scintillation fluid and therefore were weighed directly into the vials; 10 mL of liquid scintillation fluid (Packard Insta-gel) was added, the vial was vortexed, and the radioactivity was measured in the Beckman LSC for 20 min. Attempts were made to count egg yolk samples directly. However, the yolk was not miscible with the liquid scintillation fluid even after the addition of Soluene, a tissue dissolver. To determine the recovery of ^{14}C, three control albumen samples and five control samples fortified with 50 μL of 0.011 μCi/mL (1121 dpm) of [^{14}C]SMZ were prepared, mixed with Insta-gel, and counted as above. The control counts were subtracted from the sample counts during calculations. Five samples of standard [^{14}C]SMZ, containing 1121 dpm, were mixed with Insta-gel, counted, and used for the estimation of recovery. The average recovery of ^{14}C from [^{14}C]SMZ-fortified albumen was 95% with a 3% CV.

Sample Extraction and Cleanup for HPLC Analysis. Approximately 1 g each of control, fortified, and incurred albumen and yolk samples was used. For quality control purposes, control egg albumen and yolk samples were fortified with 100 μL of 0.11 μCi/mL [^{14}C]SMZ during the incurred sample analysis. The sample extraction, cleanup, and HPLC analysis conditions were similar to those reported previously (Shaikh et al., 1999) with the exception of using a flow-through radioactivity detector for monitoring of [^{14}C]sulfamethazine and its potential ^{14}C metabolites. The radioactivity detector was also fitted with pump/mixer panels along with splitter/radwaste diverter valves. Liquid scintillator (True Count) was pumped in the ratio of 3:1 with the mobile phase and mixed with the HPLC eluate to measure the radioactivity of the analyte.

RESULTS AND DISCUSSION

Total ^{14}C Residue Determination in Egg Albumen and Yolk. The depletion of the total radioactive residue (TRR) in egg albumen and yolk after the single dose of [^{14}C]SMZ to a hen is shown in Figure 1. The TRR levels in egg albumen and yolk peaked after 1 and 2 days of withdrawal period, respectively. The peak concentrations in albumen (49.1 ppm) were >4-fold greater than in yolk (11.8 ppm). On the second day of drug withdrawal, the TRR levels in yolk slightly increased, from 10.6 to 11.8 ppm. However, in the case of albumen, they significantly declined, from 49.1 to 17.4 ppm. From withdrawal day 3, the TRR levels declined even more rapidly in egg albumen and were depleted to <0.1 ppm by day 6. In contrast, the TRR levels in

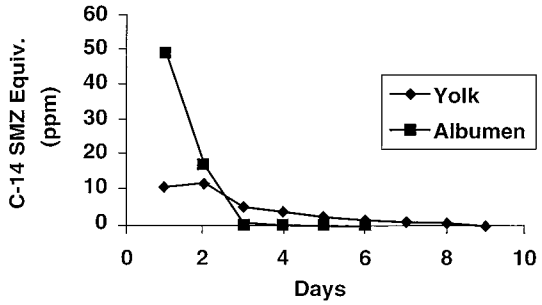

Figure 1. Depletion profile of [^{14}C]SMZ in egg albumen and yolk of a hen administered with a single dose of [^{14}C]SMZ.

Table 1. ^{14}C Residues (SMZ Equivalents in Parts per Million) in Liver, Blood, Plasma, and Muscle after Multidose Treatment

chicken	withdrawal time	liver (% CV, $n = 3$)	blood (% CV, $n = 3$)	plasma (% CV, $n = 3$)	muscle (% CV, $n = 3$)
1	1 h	159.7 (6.7)	150.0 (0.9)	138.3 (2.1)	110.5 (1.6)
2	1 day	132.3 (3.2)	110.2 (1.3)	112.2 (0.2)	76.3 (2.2)
3	2 days	85.8 (1.6)	56.1 (1.4)	55.4 (0.2)	34.4 (4.8)
4	3 days	19.7 (3.7)	3.6 (1.8)	1.7 (6.5)	0.3 (4.4)

Table 2. ^{14}C Residues (SMZ Equivalents in Parts per Million) in Egg Albumen and Yolk after Multidose Treatment to Egg-Laying Chickens

chicken	days after first dose	SMZ (ppm) in albumen	% CV ($n = 3$)	SMZ (ppm) in yolk	% CV ($n = 3$)
1	1	67	1.7	22	22.4
2	1	57	0.6	18	4.9
5	1	1.9	1.9	0.03	12.3
7	1	33.7	2.7	7.2	10.7
9	1	52.7	31.6	7.9	9.3
2	2	109	1.0	38	31.1
3	2	69.8	2.8	15.8	21.6
4	2	73.8	6.9	23.3	13.7
6	2	78.1	3.4	20.2	162.3
7	2	125.8	4.2	43.3	2.4
8	2	62.6	11.4	18.7	6.3
10	2	96.5	3.4	14.4	30.1
1	3	150.5	3.3	151.2	12.7
4	4	163.8	1.7	70.1	5.1
5	4	156.1	2.4	67.4	0.7
7	4	198.2	4.1	74.3	9.3
8	4	217.4	3.3	105.4	10.6
8	5	395.1	5.6	158.2	7.9
2	6 (WD-1)[a]	177.6	2.1	106	5.5

[a] WD-1, withdrawal day 1.

yolk declined more slowly and were depleted to 0.1 ppm by day 9. A similar depletion profile was noted (Shaikh et al., 1999) in a study in which a single dose of nonlabeled SMZ was administered to a hen. In addition, low concentrations of N_4-acetyl metabolite were found in both albumen and yolk, but none was detected in the current study. This could perhaps be due to the low specific activity of the ^{14}C-labeled N_4-acetyl metabolite. A significantly reduced level of egg production was observed during and after multidose medication of the hens, and all except one hen stopped laying eggs after the administration of the last dose. This hen also stopped laying further eggs after 1 day of the withdrawal period. Most sulfonamides are known to produce some degree of toxicity and depress egg production at high doses (North, 1984). The decrease in egg production in this study is perhaps attributed to the administered dose of sulfamethazine, which might be deemed to be high for normal egg production. Table 2 shows that during the drug treatment period, the TRR levels were severalfold greater in egg albumen (53−395 ppm) than in yolk (7.2−158 ppm) and peaked in both tissues on the last day of medication. The peak TRR concentrations in albumen and yolk were 395 and 158 ppm, respectively. As noted above in a single-dose study, the TRR levels were greater in albumen (178 ppm) than in yolk (106 ppm) after the first day of withdrawal. Geertsma et al. (1987) also reported higher concentrations of SMZ in albumen than in yolk at 1 day of withdrawal period, when a multiple dose of nonlabeled SMZ was administered to hens.

Total ^{14}C Residue Determination in Tissues and Blood. Table 1 shows the TRR concentrations in liver, muscle, blood, and plasma after multidose medication. The TRR concentrations continue to decline with time in all tissues. The maximum concentration accumulated was in liver followed by in decreasing order blood, plasma, and muscle. After 1 day of withdrawal, the TRR concentrations in liver (132 ppm) were comparable to the levels in egg (average of an amount in albumen and yolk = 142 ppm).

Determination of [^{14}C]SMZ Residues in Egg Albumen and Yolk by HPLC. In the multidose study, only one chicken laid eggs during the first 2 days of dosing and 1 day after the last dose. Therefore, the eggs from this chicken were selected for HPLC analysis. The [^{14}C]SMZ concentrations of 167 and 914 ppb equivalents were detected in egg albumen after 1 and 2 days of dosing, respectively, and no [^{14}C]SMZ residue was detected in egg yolk.

Concentrations of [^{14}C]SMZ in egg albumen and yolk, after 1 day of withdrawal, were 1076 and 588 ppb equivalents, respectively. In the single-dose study, [^{14}C]-SMZ concentrations were found only in eggs obtained after 1 day of withdrawal. They were 153 and 48 ppb equivalents in egg albumen and yolk, respectively. No metabolites were detected in samples either from multidose or from single-dose experiments. No attempts were made to determine radioactivity in protein precipitate during initial acetonitrile (ACN) extraction of egg yolk and albumen. Radioactivity in ACN extract only is reflected by HPLC analysis; therefore, no correlation could be made with radioactivity obtained from the combustion analysis. The recoveries of [^{14}C]SMZ-fortified quality control samples of egg albumen and yolk were 70 and 67%, respectively. The samples were not fortified with the ^{14}C metabolites of SMZ to determine their recoveries because none of the metabolites were detectable in incurred samples. However, the recoveries of nonlabeled SMZ and its metabolites from fortified egg albumin and yolk samples ranged from 63 to 101% with CVs of 3−9% and are reported in Shaikh et al. (1999). Figure 2a shows a typical radiochromatogram of a mixture of [^{14}C]SMZ and its metabolites, [^{14}C]glucosyl-SMZ, [^{14}C]-N_4-acetyl-SMZ, and [^{14}C]desamino-SMZ. Panels b and c of Figure 2 show radiochromatograms of egg albumen and yolk samples of eggs, respectively, obtained after 1 day of withdrawal. Albumen and yolk were from eggs collected during single- and multiple-dose studies, respectively. Only the parent [^{14}C]SMZ peak was determined in both egg albumen and yolk samples; no metabolites were detectable. The identity of the [^{14}C]-SMZ peak in unknown samples was determined by matching the retention time of pure standards of both nonlabeled and labeled SMZ. Peak heights in mAu of the radiochromatograms of [^{14}C]SMZ were converted into dpm using standards. Subsequently, the dpm

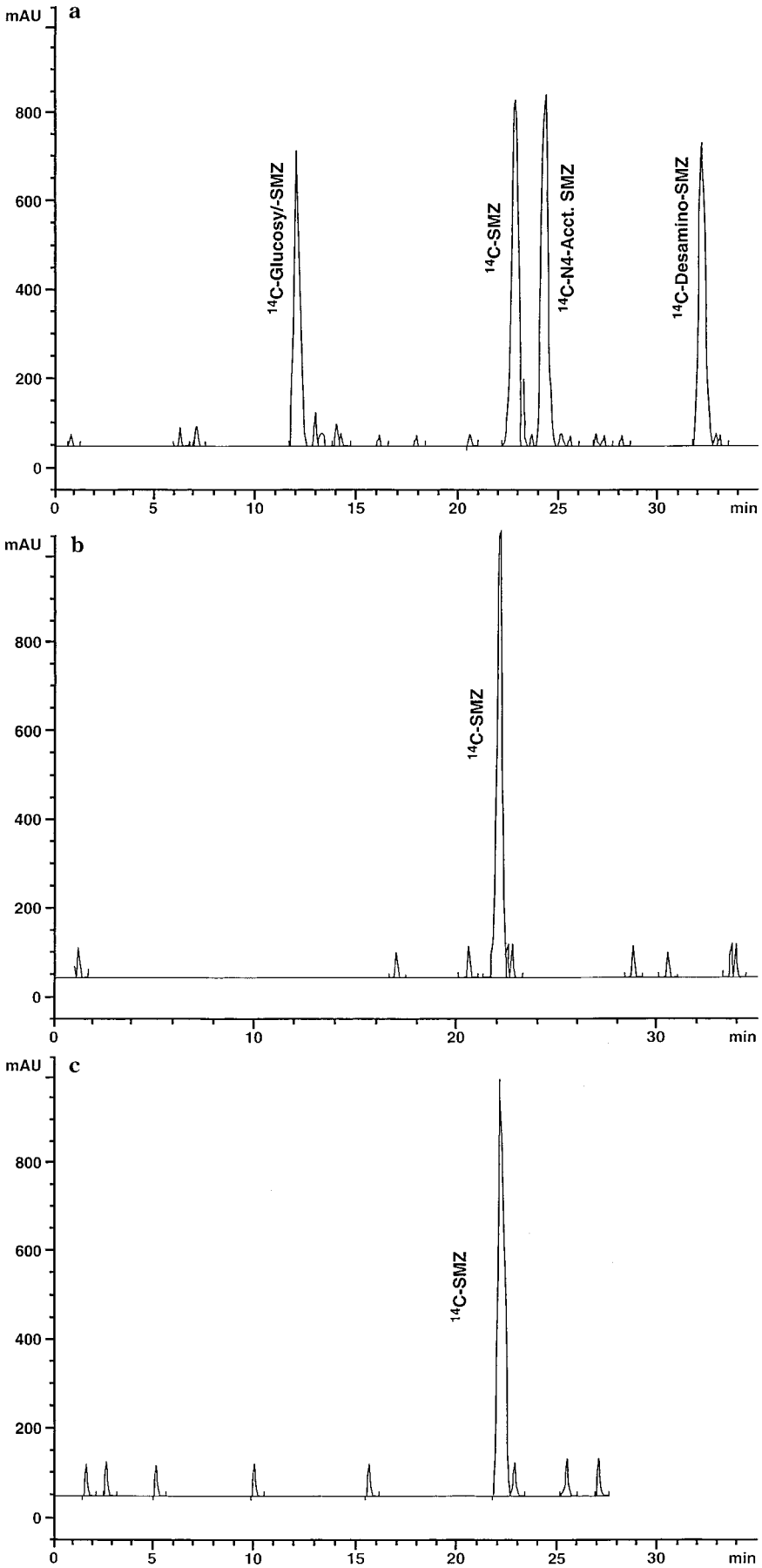

Figure 2. (a) Typical radiochromatogram of [^{14}C]SMZ and its major ^{14}C metabolite standards; (b) radiochromatogram of an incurred [^{14}C]SMZ egg albumen sample collected from a single-dose study; (c) radiochromatogram of an incurred [^{14}C]SMZ egg yolk collected from a multiple-dose study.

values were converted into parts per billion (nanograms per gram) on the basis of the specific activity of [^{14}C]-SMZ.

Conclusions. The concentration of total ^{14}C residue in albumen was higher than in yolk for the first 2 days after the single-dose treatment. However, from the third day, the levels were higher in egg yolk and also remained for a longer period than in albumen. Similarly, during the multidose treatment and 1 day after drug withdrawal, the total ^{14}C residue levels were higher in egg albumen than in yolk. After 1 day of withdrawal, the ^{14}C residue levels in liver were comparable to those transferred in eggs. The HPLC results indicate that the parent drug is the marker residue in both egg albumen and yolk. However, it should be noted that a limited number of eggs were assayed from the multiple-dose study due to a significant reduction in egg production by hens during the multidose study.

ACKNOWLEDGMENT

We are grateful to Dr. Guy Paulson for conducting animal experiments at USDA/ARS, Biosciences Research Laboratory, Fargo, ND.

LITERATURE CITED

Adams, P. E.; Feil, V. G.; Paulson, G. D. Metabolism of ^{14}C-Sulfadimethoxine in Swine. *Xenobiotica* **1996**, *9*, 921−933.

Barron, M. G.; James, M. O. Oral Bioavailability and Disposition of Sulphadimethoxine in Lobster. *Xenobiotica* **1994**, *24*, 921−931.

Code of Federal Regulations, Food & Drugs, Parts 520.2261a,b; U.S. GPO: Washington, DC, 1997.

Geertsma, M. F.; Nouws, J. F. M.; Grondel, J. L.; Aerts, M. M. L.; Vree, T. B.; Kan, C. A. Residues of Sulfadimidine and Its Metabolites in Eggs Following Oral Sulfadimidine Medication of Hens. *Vet. Q.* **1987**, *9*, 67−75.

North, M. O. Drugs and Antibiotics for Disease Control. In *Commercial Chicken Production Manual*; AVI Publishing: Westport, CT, 1984.

Paulson, G. D.; Giddings, J. M.; Lamoureux, C. H.; Manasager, E. R.; Struble, C. B. The Isolation and Identification of ^{14}C-Sulfamethazine Metabolites in the Tissues and Excreta of Swine. *Drug Metab. Dispos.* **1981**, *9*, 142−146.

Paulson, G. D.; Mitchel, A. D.; Zaylskie, R. G. Identification and quantitation of Sulfamethazine Metabolites by HPLC and GC-MS. *JAOAC Int.* **1985**, *68*, 1000−1006.

Roudaut, B. Residues of Sulphonamides in Eggs Following Oral Medication of Laying Hens. *Proceedings of the EuroResidue II Conference*; Utrecht University Press: Veldhoven, The Netherlands, 1993; Vol. 2, pp 591−595.

Shaikh, B.; Rummel, N.; Donoghue, D. Determination of Sulfamethazine and Its Major Metabolites in Egg Albumen and Egg Yolk by High Performance Liquid Chromatography. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 2651−2662.

Received for review April 26, 2000. Revised manuscript received July 19, 2000. Accepted July 24, 2000.

JF000519E