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Hapten Synthesis and Development of Polyclonal Antibody-Based Multi-Sulfonamide Immunoassays

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This paper reports the synthesis of five sulfonamide derivatives, the production of broad-specificity polyclonal antibodies for immunoassay of sulfonamides, and the analysis of milk samples by developed assay. The three-step synthesis procedure reported in most of the literature was adopted and modified in this study. In the procedure, the purification of the intermediate was avoided and the time of synthesis was shortened from >20 to 6−9 h with improved yields. This method is generally applicable to the synthesis of haptens containing the common structure of sulfonamides. Three haptens were coupled to keyhole limpet hemocyanin, and polyclonal antibodies were obtained from rabbits immunized with these conjugates. Using the antibodies obtained, from one of these was developed an enzyme-linked immunosorbent assay (ELISA) based on the competition between free sulfonamides and the hapten−horseradish peroxidase (HRP) conjugates. The hapten−HRP conjugate giving the best competitive results and 11 **s**tructurally different sulfonamides showed 50% inhibition at concentrations of <100 ng mL^{-1}. After removal of the protein with acetone, milk samples were analyzed by ELISA directly; a matrix effect could be avoided when a 1:20 dilution with phosphate-buffered saline was used, and 104−131% recoveries of spiked samples were obtained. The developed immunoassay is suitable to determine sulfisozole, sulfathiazole, sulfameter, sulfamethoxypyridazine, sulfapyridine, and sulfamethizole below the maximum residue limit in milk (100 ng mL^{-1} of total sulfonamides) rapidly and reliably.

KEYWORDS: Sulfonamides; milk; ELISA; hapten; polyclonal antibodies

INTRODUCTION

Sulfonamides are widely used for therapeutic and prophylactic purposes in humans and other animals and sometimes as additives in animal feed (*1*). These synthetic compounds are derivatives of sulfanilamide with an aromatic amino group at the N_4-position and differing in the substitution at the N_1-position. The backbone and the structures of sulfonamides studied in this work are shown in **Figure 1**.

If the proper withdrawal periods are not observed before slaughtering or milking of the medicated animals, meat or milk from these animals may be contaminated with residual sulfonamides (*2*). The presence of sulfonamide residues in food is considered harmful to consumers. In the European Union, Canada, and the United States, the maximum residue limit (MRL) of total sulfonamides in edible tissues is 100 $\mu g\ kg^{-1}$ (*3*, *4*), and it is 20 $\mu g\ kg^{-1}$ in Japan (*5*).

Currently, Integral Production Chain Systems demand faster on-site (farmhouses) and/or on-line (slaughterhouses) test systems. Immunoassays are capable of detecting low amounts of residues in many samples rapidly. An enzyme-linked immunosorbent assay (ELISA) has been developed for detecting several of the sulfonamides. Antibodies for the detection of individual sulfonamides were produced by hapten conjugates, which were prepared by modification at the N_4-position, leaving the specific structure of the sulfonamides unchanged. Different immunoassay kits for the detection of sulfonamide residues in food have been marketed using these antibodies. The broad-specificity antibodies obtained by using sulfonamide derivatives, differing at the N_1-position, leaving the common structure of the sulfonamides exposed, were expected to provide a general assay for groups of sulfonamides. More effort is now being directed to developing such broad-specificity antibodies for detecting groups of sulfonamides.

In the past 20 years, several authors (*5*−*9*, *14*) have designed and synthesized several sulfonamide haptens that all had carboxyl groups at the N_1-position and kept the common structure of sulfonamides unchanged. These haptens were mostly synthesized according to a three-step procedure, and the synthetic route was time-consuming, laborious, and low in yield. In this paper, this three-step procedure was modified with improvement in the performances.

Sheth and Sporns (*6*) immunized rabbits with a sulfathiazole derivative (*N*1-[4-(carboxymethyl)-2-thiazolyl]-sulfonamide) linked

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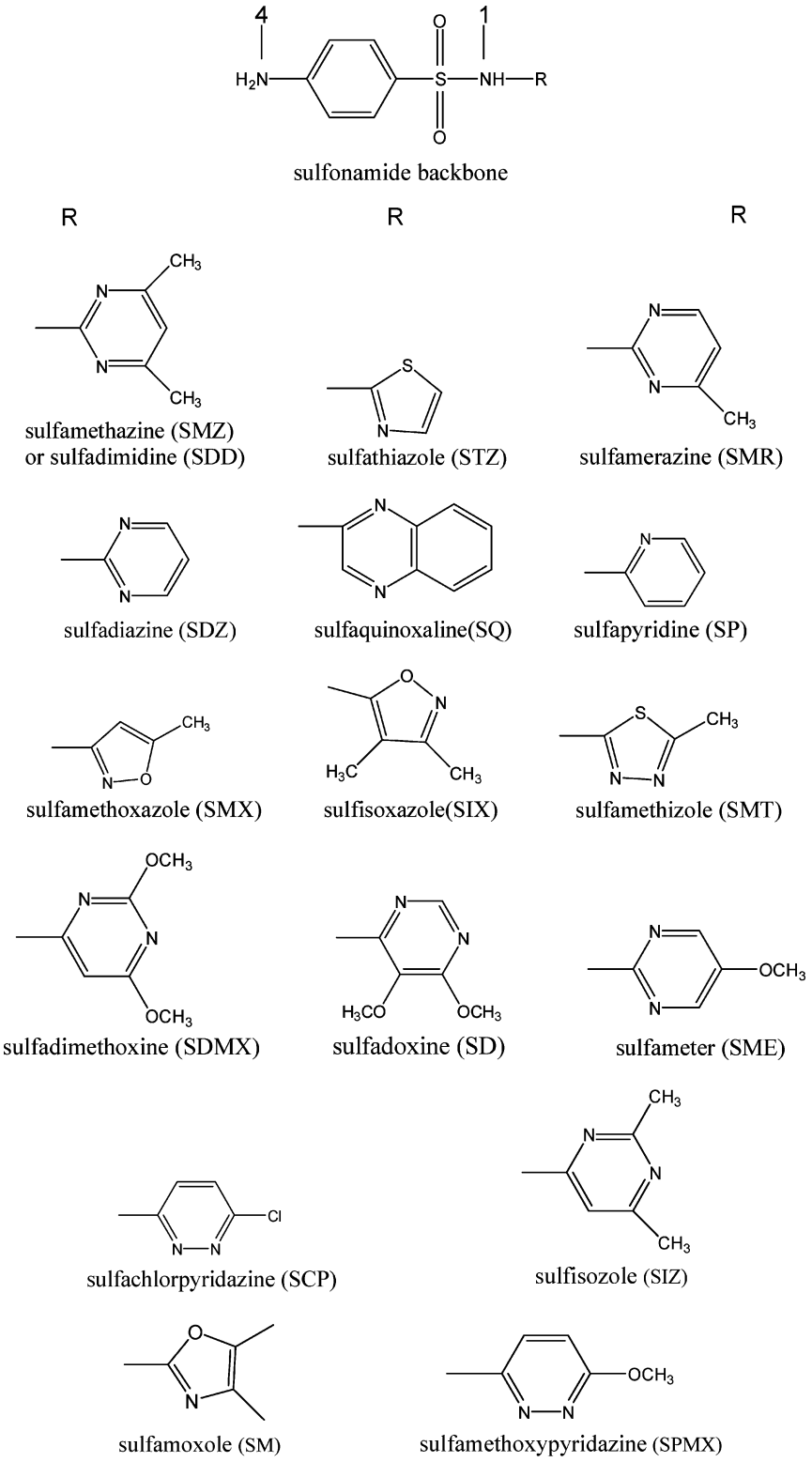

Figure 1. Structures of the sulfonamides studied in this work.

to keyhole limpet hemocyanin (KLH). The polyclonal antibodies recognized nine sulfonamides showing 50% inhibition at a concentration of $<5\ \mu g\ mL^{-1}$. Assil et al. (*10*) synthesized another sulfonamide derivative (*N*1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl]-azo-2-pyridyl]-sulfanilamide). The purified polyclonal antibody obtained showed 50% inhibition with seven sulfonamides at concentrations of $<10\ \mu g\ mL^{-1}$. The first published study on broad-specificity monoclonal antibodies was from Muldoon et al. (*11*). After immunization with an *N*-sulfanilyl-4-aminobenzoic acid−protein conjugate, only one monoclonal antibody was obtained that recognized eight sulfonamides at levels of $<10\ \mu g\ mL^{-1}$. Haasnoot et al. (*7*) used the sulfonamide derivatives of Sheth and Sporns (*6*) and Assil et al. (*10*) to induce monoclonal antibodies, and one antibody showed 50% inhibition with 18 tested sulfonamides at values of $<10\ \mu g\ mL^{-1}$ and with eight at a concentration of $<0.1\ \mu g\ mL^{-1}$.

Korpimaki et al. (*12*) used protein engineering to improve the broad specificity of sulfonamide antibodies. The new mutants

recognized different sulfonamides with affinities sufficient for the detection of all 13 tested sulfonamides below 100 ng mL^{-1}.

The aim of this study was the production of broad-specificity polyclonal antibodies using various synthesized hapten−KLHs as immunogens. With such antibodies a sensitive, broad-specificity immunoassay was developed that could detect several sulfonamides at relevant levels.

MATERIALS AND METHODS

Reagents. *N*-Acetylsulfanilyl chloride, 4-aminobenzoic acid, 2-amino-4-thiazoleacetic acid, 6-aminonicotinic acid, and 4-aminobutanoic acid were obtained from Fluka-Sigma-Aldrich Co. Pyridine and ethanol were of analytical grade, dried, and purified before use. Horseradish peroxidase (HRP), KLH, bovine serum albumin (BSA), reagent grade 3,3′,5,5′-tetramethylbenzidine (TMB), fish skin gelation (FG), and Freund's complete and incomplete adjutants were purchased from Sigma (St. Louis, MO). Protein A−Sepharose 4B was purchased from Amersham (Uppsala, Sweden). HPLC grade methanol were obtained from Merck (Darmstadt, Germany). Reagent grade 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride) (EDC) and hydrogen peroxide were from Sigma.

Solutions. Phosphate-buffered saline (PBS; 10 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, pH 7.5), PBS with 0.05% Tween 20 (PBS-T), coating buffer (CB; 50 mmol L^{-1} sodium carbonate buffer, pH 9.6), and TMB substrate solution [prepared by adding 3.3 mg of TMB in 250 μL of DMSO to 25 mL of phosphate−citrate buffer (0.1 mol L^{-1} citric acid + 0.2 mol L^{-1} Na_2HPO_4; pH 4.3) containing 3.25 μL of a 30% H_2O_2 solution] were used.

Instrumentation. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Bruker AV-300 and Bruker AV-400 spectrometers, and the chemical shifts were expressed in parts per million (δ scale) using tetramethylsilane as an internal standard. Infrared spectroscopy (IR) spectra were performed using a Bruker Vector 22 spectrometer. The ESI-MS spectra were obtained on a Thermo-Finnegan LCQ Advantage HPLC with mass spectrometry (HPLC-MS) instrument. Thin-layer chromatography (TLC) was performed on silica gel G detected by an ultraviolet−visible (UV) detector. For column chromatography, silica gel 200−300 mesh was used. Polystyrene 96-well microwell plates were from Nunc (Roskilde, Denmark), and the microplate washer was from Bio-Rad (Hercules, CA). Immunoassay absorbance was read with a Multiscan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland) in dual wavelength mode (450−650 nm).

Haptens Synthesis. The structures of the haptens designed are shown in **Figure 2**.

General Procedure for the Preparation of Haptens D1−D5. N-Acetylsulfanilyl chloride (0.5328 g, 2.28 mmol) was dissolved in dry pyridine (5 mL), and a solution of acid or ester including an amino group (2.12 mmol) in 6 mL of dry pyridine was dropwise added under N_2 atmosphere with stirring. After addition, the reaction mixture was refluxed at 90 °C for 2−3 h, and the reaction was monitored by TLC. Then 20 mL of 2 M NaOH was added, and the pyridine was removed under reduced pressure. The reaction mixture was refluxed for an additional 2−3 h, and the reaction was monitored by TLC. Afterward, the mixture was cooled to room temperature, and the pH was adjusted to 4.0 with concentrated HCl. The mixture was extracted with ethyl acetate (3 × 30 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was further purified on a silica gel column (200−300 mesh), and the elution conditions were established according to the TLC results.

Synthesis of Hapten [2-(4-Aminobenzenesulfonylamino)-1,3-thiazol-4-yl]acetic Acid (D1)]. Synthesis of (2-Amino-1,3-thiazol-4-yl)acetic Acid Ethyl Ester (M1). Ethanol (18.4 mL) and concentrated HCl (3.7 mL) were added to 1.00 g of 2-amino-4-thiazoleacetic acid (6.3 mmol) with stirring. The mixture was heated under reflux for 2−3 h with stirring, after which the solvent was removed under reduced pressure. The residue was dissolved in 19 mL of distilled water and adjusted to pH 8.0 with 6.5 mol L^{-1} NH_4OH. The mixture was extracted with ethyl acetate (3 × 20 mL), and the organic phase was dried over anhydrous Na_2SO_4. The extract was evaporated under reduced pressure, giving a yellow powder (0.6946 g, 59%), the (2-amino-1,3-thiazol-4-yl)acetic acid ethyl ester: TLC [dichloromethane/MeOH (30:1)] R_f = 0.45; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.341 (s, 1H, CH_{ar-S},), 5.166 (br, 2H, NH_2), 4.180 (q, 2H, J = 7.2 Hz, OCH_2CH_3), 3.560 (s, 2H, $COCH_2$), 1.270 (t, 3H, J = 7.2 Hz, CH_3); MS (ESI) [found, m/z 187.12 $[M + H]^+$; calcd for $C_7H_{10}N_2O_2S$: M, 186]; IR (KBr, cm^{-1}) 3420, 3282, 3144, 2990, 1717, 1626, 1525, 1474, 1448, 1423, 1371, 1337, 1311, 1265, 1172, 1130, 1033, 694.

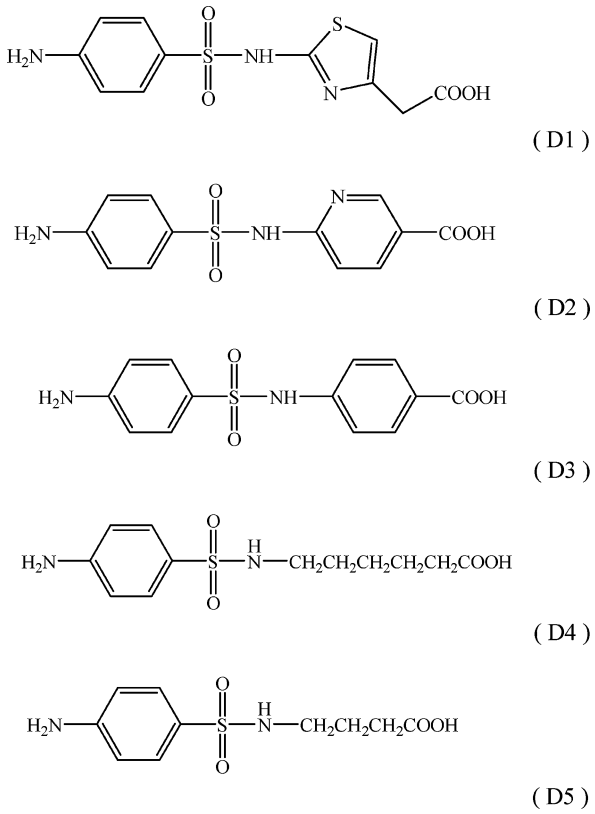

Figure 2. Chemical structures of D1–D5 haptens synthesized.

Synthesis of [2-(4-Aminobenzenesulfonylamino)-1,3-thiazol-4-yl]acetic Acid (D1). The above-described general procedure was applied to prepare hapten D1; 0.3963 g of (2-amino-1,3-thiazol-4-yl)acetic acid ethyl ester (2.12 mmol) was used. The residue was a brown-red oil and then purified in silica gel columns using methanol/chloroform/ice acetic acid (1:20:0.04) as eluant, giving product D1 (0.1654 g, 24.9% yield, yellow powder): TLC [methanol/chloroform/ice acetic acid (1:10:0.02)]: R_f = 0.35; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.392 (br, 2H, COOH, SO_2NH), 7.457 (d × t, 2H, J = 8.7 Hz, 2.3 Hz, CH_{ar}), 6.584 (d × t, 2H, J = 8.7 Hz, 2.3 Hz, CH_{ar}), 6.533 (s, 2H, NH_2), 3.534 (s, 1H, CH-$_S$), 1.924 (s, 2H, CH_2COOH); MS (ESI) [found: m/z 314.12 $[M + H]^+$, calcd for $C_{11}H_{11}N_3O_4S_2$, M, 313.35]; IR (KBr, cm^{-1}) 3476, 3383, 3108, 2925, 1717, 1697, 1624, 1595, 1541, 1501, 1419, 1340, 1294, 1269, 1241, 1186, 1138, 1086, 1023, 885, 829, 738, 715, 691, 620, 572, 552.

Synthesis of Hapten 6-(4-Aminobenzenesulfonylamino)nicotinic Acid (D2). Synthesis of 6-Aminonicotinic Acid Ethyl Ester (M2). M2 was prepared as the route of M1 preparation, and a white powder was obtained (0.3583 g, 75%): TLC [dichloromethane/methanol (16:1)] R_f = 0.66; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.734 (d, 1H, J = 2.1 Hz, CH_{ar-N}), 8.023 (dd, 1H, J = 2.1, 9.0 Hz, CH_{ar-CCO}), 6.475 (d, 1H, J = 9.0 Hz, CH_{ar-CNH_2}), 4.954 (br, 2H, NH_2), 4.341 (q, 2H, J = 7.2 Hz, CH_3CH_2O), 1.372 (t, 3H, J = 7.2 Hz, CH_3CH_2O); MS (ESI) [found: m/z 166.37 $[M]^+$, calcd for $C_8H_{10}N_2O_2$, M, 166]; IR (KBr, cm^{-1}) 3414, 3321, 3142, 2977, 1692, 1652, 1602, 1515, 1444, 1370, 1277, 1138, 950, 836, 783.

Synthesis of 6-(4-Aminobenzenesulfonylamino)nicotinic Acid (D2). The above-described general procedure was applied to prepare the hapten D2. For that, 0.3534 g of 6-aminonicotinic acid ethyl ester (2.12

mmol) was used. The residue was a yellow powder. Further purification in silica gel columns using methanol/chloroform/ice acetic acid (1:20:0.04) as eluant finally gave the product D2 (0.1564 g, 25.2% yield, yellowish powder): TLC [methanol/chloroform/ice acetic acid (5:5:0.04)] $R_f = 0.76$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.167 (br, 1H, COOH), 8.581 (s, 1H, SO_2NH), 8.548 (d, 1H, $J = 4.4$ Hz, CH_{ar-N}), 8.057 (d × d, 1H, $J = 8.8$ Hz, 2.4 Hz, CH_{ar-CCO}), 7.537 (d, 2H, $J = 8.8$ Hz, CH_{ar}), 7.070 (d, 1H, $J = 8.8$ Hz, CH_{ar-CNH}), 6.532 (d, 2H, $J = 8.8$ Hz, CH_{ar}), 6.029 (s, 2H, NH_2); MS (ESI) [found: *m*/*z* 294.17 $[M + H]^+$, calcd for $C_{12}H_{11}N_3O_4S$, M, 293]; IR (KBr, cm^{-1}) 3474, 3375, 3234, 1707, 1635, 1595, 1538, 1505, 1383, 1298, 1276, 1139, 1058, 959, 837, 770, 681, 666, 583, 554.

Synthesis of Hapten 4-(4-Aminobenzenesulfonylamino)benzoic Acid (D3). The above-described general procedure was applied to prepare the hapten D3. For that, 0.2900 g of 4-aminobenzoic acid (2.12 mmol) was used. The residue was a yellow oil, and further purification in silica gel columns using methanol/chloroform/ice acetic acid (1:40:0.08) as eluant finally gave the product D3 (0.4445 g, 71.8% yield, yellow powder): TLC [methanol/chloroform/ice acetic acid (1:20:0.04)] $R_f = 0.25$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.803 (d, 2H, $J = 8.0$ Hz, CH_{ar}), 7.479 (d, 2H, $J = 8.0$ Hz, CH_{ar}), 7.162 (d, 2H, $J = 8.0$ Hz, CH_{ar}), 6.575 (d, 2H, $J = 8.4$ Hz, CH_{ar}), 6.071 (s, 2H, NH_2); MS (ESI) [found: *m*/*z* 606.86 $[2M + Na]^+$, calcd for $C_{13}H_{12}N_2O_4S$, M, 292]; IR (KBr, cm^{-1}) 3494, 3464, 3391, 3373, 3167, 2930, 1698, 1633, 1608, 1595, 1505, 1460, 1407, 1324, 1291,1235, 1187, 1148, 1092, 1018, 912, 834, 769, 718, 678, 658, 578, 548.

Synthesis of Hapten 6-(6-Aminobenzenesulfonylamino)hexanoic Acid (D4). The above-described general procedure was applied to prepare the hapten D4. For that, 0.2777 g of 6-aminohexanoic acid (2.12 mmol) was used. The residue was a yellow oil and purified in silica gel columns using methanol/chloroform/ice acetic acid (1:20:0.04 and 1:10:0.02) as eluant, finally giving the product D4 (0.2158 g, 35.6% yield, milky powder): TLC [methanol/chloroform/ice acetic acid (1:10:0.02)] $R_f = 0.61$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.957 (br, 1H, COOH), 7.403 (d × t, 2H, $J = 8.7$ Hz, 2.4 Hz, CH_{ar}), 7.065 (t, 1H, $J = 6.0$ Hz, SO_2NH), 6.607 (d × t, 2H, $J = 8.7$ Hz, 2.3 Hz, CH_{ar}), 5.923 (s, 2H, NH_2), 2.519 (d × d, 2H, $J = 6.8$ Hz, 6.3 Hz, $NHCH_2$), 2.156 (t, 2H, $J = 7.4$ Hz, CH_2COOH), 1.471−1.159 (m, 6H, $CH_2CH_2CH_2$); MS(ESI) [found: *m*/*z* 595.10 $[2M + Na]^+$, calcd for $C_{12}H_{18}N_2O_4S$, M, 286]; IR(KBr, cm^{-1}) 3413, 3326, 3126, 2937, 2859, 1699, 1627, 1596, 1502, 1467, 1429, 1304, 1250, 1231, 1197, 1143, 1095, 1036, 1013, 985, 936, 846, 678, 581, 563, 546.

Synthesis of Hapten 6-(4-Aminobenzenesulfonylamino)butanoic Acid (D5). The above-described general procedure was applied to prepare the hapten D5. For that, 0.2184 g of 4-aminobutanoic acid (2.12 mmol) was used. The residue was a yellow oil and purified in silica gel columns using methanol/chloroform/ice acetic acid (1:20:0.04, 1:10:0.02 and 1:5:0.01) as eluant, finally giving the product D5 (0.1538 g, 28.1% yield, yellowish powder): TLC [methanol/chloroform/ice acetic acid (1:10:0.02)] $R_f = 0.26$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.109 (br, 1H, COOH), 7.423 (d, 2H, $J = 8.7$ Hz, CH_{ar}), 7.151 (t, 1H, $J = 5.7$ Hz, SO_2NH), 6.630 (d, 2H, $J = 8.7$ Hz, CH_{ar}), 5.957 (s, 2H, NH_2), 2.683 (d × d, 2H, $J = 6.0$ Hz, 6.8 Hz, 6.3 Hz, $NHCH_2$), 2.226 (t, 2H, $J = 7.2$ Hz, CH_2COOH), 1.594 (m, 2H, $CH_2CH_2CH_2$); MS (ESI) [found: *m*/*z* 258.87 M + H]$^+$, calcd for $C_{10}H_{14}N_2O_4S$, M, 258]; IR (KBr, cm^{-1}) 3413, 3336, 3127, 2950, 2622, 1907, 1699, 1633, 1596, 1501, 1432, 1305, 1261, 1216, 1188, 1147, 1090, 1012, 965, 896, 841, 682, 577, 548, 509.

Preparation of Enzyme and Protein Conjugates. The sulfonamide derivatives were attached to the carrier proteins (KLH, OA) or HRP using a mixed anhydride method using EDC without isolation of intermediates. Haptens D1, D3, and D4 were coupled to KLH for use as immunogens. All five haptens were coupled to OA as coating proteins, coupled to HRP as enzyme tracers, respectively.

The carboxylic acid of the hapten (0.025 mmol) and 0.05 mmol of EDC were dissolved in 1 mL of dry DMF. Then mixture was added dropwise with mixing to 2 mL of solution of 10 mg of protein (KLH, OA, or HRP) in 130 mmol L^{-1} sodium bicarbonate (pH 8.1). After 4−6 h of mixing at room temperature on a mixing wheal, another 4.7 mg of EDC was added. After mixing overnight at 4 °C on the mixing wheel, the mixture was dialyzed against PBS.

Antibody Production. Antibodies were produced in rabbits as described by Wang et al. (*13*). Six white rabbits (two for each immunogen) were immunized by intradermal and intramuscular injections of the immunogens D1−KLH, D3−KLH, and D4−KLH. IgG from the antisera was purified by Protein A−Sepharose 4B affinity chromatography.

Screening of Antisera. Antisera titers, antibody coating quantity, and enzyme conjugate dilution factors were optimized to give absorbance values ranging from 0.7 to 1.2 in the absence of analytes using a checkerboard titration. The antisera titers were tested for D1−KLH on D1−OA coating, for D3−KLH on D3−OA coating, and for D4−KLH on D4−OA coating using antigen-coated indirect ELISA format, and the antisera were diluted from 1/1000 to 1/200000 to microtiter plates coated with 1 μg per well of the hapten−OA conjugate. Similarly, antibody coating quantity and enzyme conjugate dilution factors were tested on an antibody-coated direct ELISA format using enzyme conjugate dilutions from 1/1000 to 1/100000 and plates coated with 0.5, 1, or 1.5 μg per well of the antibodies

Indirect ELISA. Flat-bottom polystyrene ELISA plates were coated with OA−hapten (D1−D5) conjugates at 1 μg per well in 100 μL of CB and incubated overnight at room temperature. Plates were then washed three times with 10 mmol L^{-1} PBS-T, and unbound active sites were blocked with 200 μL of 1% BSA/PBS per well for 1 h. After the plate had been washed four times, 100 μL of the appropriate sera dilution in PBS was added per well and incubated for 1 h at room temperature. After four washings, plates were incubated for 1 h with peroxidase-labeled goat anti-rabbit immunoglobulins diluted 1:10000 in PBS (100 μL per well). After five washings, the HRP tracer activity was measured by adding 150 μL per well of TMB substrate solution. The enzymatic reaction was stopped after 30 min by adding 2.5 mol L^{-1} H_2SO_4 (50 μL per well), and the absorbance was read in dual-wavelength mode (450 nm as test and 650 nm as reference).

Direct ELISA. Polystyrene ELISA plates were coated with purified antibodies at 0.5, 1, or 1.5 μg per well in 100 μL of CB, incubated overnight at room temperature. Plates were then washed three times with 10 mmol L^{-1} PBS-T, and unbound active sites were blocked with 200 μL of 1% BSA/PBS per well for 1 h. After the plate had been washed four times, for competitive assays, 100 μL of standards in PBS and 100 μL of HRP−haptens in PBS were then added to each well and incubated for 1 h at room temperature. After five washings, the HRP tracer activity was measured by adding 150 μL per well of TMB substrate solution. The enzymatic reaction was stopped after 30 min by adding 2.5 mol L^{-1} H_2SO_4 (50 μL per well), and the absorbance was read in dual-wavelength mode (450 nm as test and 650 nm as reference).

Sample Preparation. Milk samples were bought from local markets. Before the spike and recovery studies, each test sample was verified to contain sulfonamides at <10 ng mL^{-1} by HPLC. For a spiking study, 20 mL milk samples were spiked with a single sulfonamide dissolved in methanol at different levels, thoroughly mixed, and then centrifugated for 10 min at 3000 rcf. After the fat layer had been discarded, 20 mL of acetone was added and shaken by hand, then centrifugated for 10 min at 3000 rcf. The upper clear liquid was diluted with PBS and analyzed.

The sample extracts were appropriately diluted before ELISA analysis, and 1% BSA, 0.5% FG, or 0.05% Tween 20 was added to the PBS to avoid the sample matrix effect.

Instrumentation for HPLC Analysis. The test sample was verified using a Shimadzu (Tokyo, Japan) HPLC equipped with an LC-10AT vp pump with a Hamilton injector (25 μL loop), a DGU-12A online degasser, and a CTO-10AS vp column oven. A C_{18} reversed-phase column (15 cm × 4.6 mm i.d., 5 μm) was used. The analyses were performed at 270 nm, and the mobile phase was methanol/water (28:72) (water pH value was adjusted to 3.5 before it was mixed with methanol) at a flow rate of 1.0 mL min^{-1}. The temperature of the column oven was 35 °C.

RESULTS AND DISCUSSION

Hapten Synthesis. Five different sulfonamide haptens (D1−D5) were previously reported for the detection of sulfonamides

Table 1. Titers of the Antisera during the Immunization

bleed	D1–KLH–1	D1–KLH–2	D3–KLH–1	D3–KLH–2	D4–KLH–1	D4–KLH–2
first	6000	2000	64000	64000	died	3000
second	died	1000	80000	160000		8000
third		1000	died	200000		20000
fourth		1000		100000		20000

Table 2. Optimization of the Coating Antibody Quantity and Enzyme Conjugate Dilution Factors

	D3–KLH–2			D4–KLH–2			D1–KLH–2		
	0.5 μg/well	1.0 μg/well	1.5 μg/well	0.5 μg/well	1.0 μg/well	1.5 μg/well	0.5 μg/well	1.0 μg/well	1.5 μg/well
D1–HRP	<1000	<1000	<1000	<1000	<1000	<1000	<1000	<1000	<1000
D2–HRP	2000	8000	10000	<1000	<1000	<1000	<1000	<1000	<1000
D3–HRP	10000	40000	40000	<1000	<1000	<1000	<1000	<1000	<1000
D4–HRP	2000	8000	10000	1000	3000	3000	<1000	<1000	<1000
D5–HRP	<1000	<1000	<1000	<1000	<1000	<1000	<1000	<1000	<1000

(*5*−*8*, *11*, *12*). They conjugated the haptens with carrier protein through the R group and produced group-specific antibodies. In previous works, these haptens were synthesized using three steps, namely, esterification of carboxylic acid compounds, condensation with *N*-acetylsulfanilyl chloride, and then basic hydrolysis to give the target molecule product. This reported method has two disadvantages: First, overall reaction times of D1−D3 described by Pastor-Navarro et al. were over 22, 44, and 31 h, respectively. The second step was the most time-consuming step and required >20 h, with reaction first in an ice bath and then at room temperature. Second, the procedure was more laborious because the product in each step had to be separated and purified.

In this work, we adopted and integrated the three-step procedure reported in most of the literature. Because the third step also took place in basic conditions, we modified the above method; namely, the pyridine in the product of the second step was evaporated, NaOH solution was added, and the mixture was refluxed to hydrolyze. This route only needed 6−9 h under the condition of reflux, which saved reaction time.

The raw materials of haptens D3, D4, and D5 could dissolve in pyridine, so they were used to synthesize these haptens directly and the step of esterification was left out. The synthesis of haptens D1 and D2 using this method gave poor yield, perharps due to the poor solubility of the raw materials (2-amino-4-thiazoleacetic acid and 6-aminonicotinic acid). Therefore, these raw materials were esterified first, and then the esters were used to synthesize these haptens. The yields of the synthesized sulfonamide derivatives [2-(4-amino-benzenesulfonylamino)-1,3-thiazol-4-yl]acetic acid (D1), 6-(4-aminobenzenesulfonylamino)nicotinic acid (D2), 4-(4-amino-benzenesulfonylamino)benzoic acid (D3), 6-(6-aminobenzene-sulfonylamino)hexanoic acid (D4), and 6-(4-aminobenzene-sulfonylamino)butanoic acid (D5) were 24.9, 25.2, 71.8, 35.6, and 28.1%, respectively. Although the final chromatography was laborious, this step assured the purification of the end product and led to the good quality of the antibodies.

Antibody Production and Screening of Antisera. Table 1 shows the optimal results of the antisera titers (the dilution of a serum containing a specific antibody at which the solution retains the minimum level of activity needed to neutralize or precipitate an antigen) during the immunization. The titers from the first three bleeds for D1−KLH−2, D3−KLH−2, and D4−KLH−2 were 1000, 100000, and 20000, respectively.

The results of optimization of antibody coating quantity and enzyme conjugate dilution factors using direct ELISA are shown in **Table 2**. This shows that the antibody of D1−KLH−2 did not recognize all five enzyme conjugates and that the antibody of D4−KLH−2 could recognize only the D4−HRP conjugate, but the dilution factors were too low. The antibody of D3−KLH−2 recognized D2−HRP, D3−HRP, and D4−HRP with relatively high dilution factors. To balance the absorbance value and save the antibody, we selected the coating antibody with a coating quantity of 1.0 μg per well, and the corresponding enzyme conjugate dilution factors were 8000, 40000, and 8000 for D2−HRP, D3−HRP, and D4-HRP, respectively.

Analytical Characteristics of the Sulfonamide ELISA. The broad specificity of the developed ELISA was evaluated by determining the cross-reactivity with a set of sulfonamides. The concentrations of analytes giving 50% inhibition of color development (IC_{50}) and cross-reactivity were determined for different sulfonamides in buffer solution, and the values obtained are given in **Table 3**. The results showed that direct ELISA developed with the antibody against D3−KLH and the D2−HRP conjugate showed high sensitivity and good broad specificity. Using this combination, the IC_{50} values of sulfisoxazole, sulfathiazole, sufameter, sulfapyridine, sulfamethoxypyridazine, sulfachlorpyridazine, sulfamethizole, sulfamerazine, sulfadoxine, sulfadiazine, and sulfaquinoxaline were below 100 ng mL^{-1} in buffer solution. These results were generally more sensitive than those of polyclonal antibodies reported previously by other researchers: Sheth and Sporns (*6*) reported IC_{50} values of 9 sulfonamides ofwere <5 μg mL^{-1}; Assil et al. (*10*) reported IC_{50} values of 7 sulfonamides of <10 μg mL^{-1}), better than those for monoclonal antibodies reported by Muldoon et al. (*11*) (IC_{50} values of 8 sulfonamides were <10 μg mL^{-1}); Haasnoot et al. (*7*) reported IC_{50} values of 18 tested sulfonamides of <10 μg mL^{-1} and of 8 sulfonamides of <0.1 μg mL^{-1} and equal to those reported by Korpimäki et al. (*12*) (could detect all 13 tested sulfonamides below 100 ng mL^{-1}). The cross-reactivities for the 11 sulfonamides were between 1.4 and 100%.

The intra-assay and interassay reproducibilities were determined to study the assay precision. The variations of percent inhibition for 20, 10, 5, 2.5, 1.25, 0.63, and 0.31 ng mL^{-1} of sulfisozole tested three times on the same day were 3.8, 3.1, 4.2, 7.5, 15.8, 24.4, and 31.1%, respectively. The assay of the same material run over 6 months gave deviations from the means of 5.9, 9.2, 11.3, 11.9, 29.5, and 35.7%. The average detection limit for buffer curves was defined as the concentration of sulfonamides causing 15% inhibition of color development. Using this criterion, the limit of detections (LOD) for sulfisozole, sulfathiazole, sufameter, sulfamethoxypyridazine, sulfapyridine,

Table 3. Assay Sensitivities of Antibody Obtained from D3–KLH-Immunized Rabbit for 16 Sulfonamides Using Different Antigen–HRP Conjugates

	D2–HRP IC_{50} (ng mL^{-1})	cross-reactivity (%)	D3–HRP IC_{50} (ng mL^{-1})	cross-reactivity (%)	D4–HRP IC_{50} (ng mL^{-1})	cross-reactivity (%)
sulfisozole	1.3	100	12	100	18	100
sulfathiazole	2	65	15	80	25	72
sufameter	3.2	41	25	48	19	95
sulfamethoxypyridazine	8.4	15	20	60	32	56
sulfapyridine	10	13	25	48	28	64
sulfamethizole	12.8	10	130	9	125	14
sulfachlorpyridazine	14	9	55	22	68	26
sulfamerazine	19	7	85	14	155	12
sulfadoxine	31	4	150	8	>200	<9
sulfadiazine	32	4	150	8	190	9
sulfaquinoxaline	90	1.4	175	7	>200	<9
sulfamethazine	130	1	200	6	>200	<9
sulfamethoxazole	135	1	175	7	>200	<9
sulfamoxole	150	0.9	200	6	>200	<9
sulfadimethoxine	190	0.7	80	15	>200	<9
sulfisoxazole	>200	<0.7	>200	<6	>200	<9

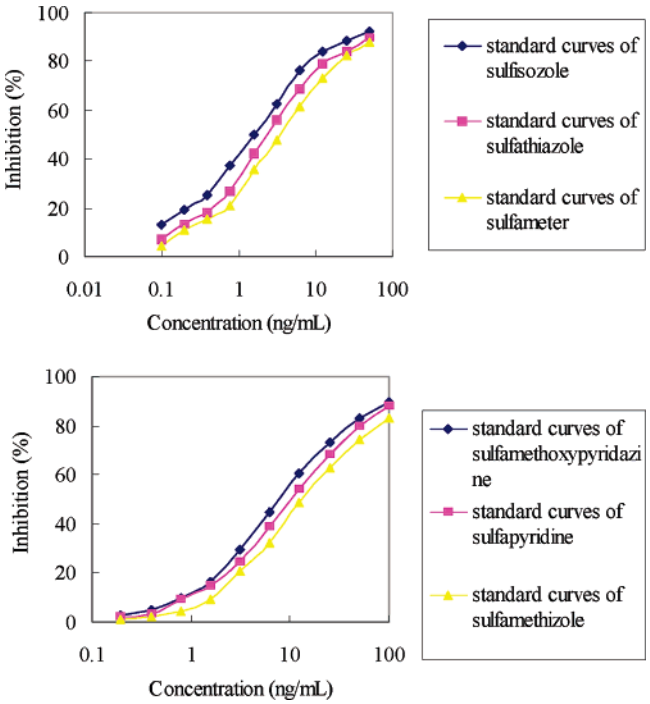

Figure 3. Standard curves of six sulfonamides detected in milk below the MRL.

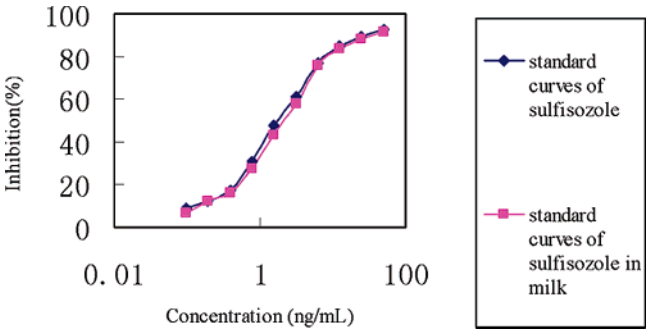

Figure 4. Standard curves of sulfisozole.

Table 4. Recoveries of Sulfonamides Spiked at Three Concentrations in Milk Samples

analyte	fortification level (ng mL^{-1})	mean ± SD (ng mL^{-1})	recovery (%)	CV (%)
sulfisozole	50	52.47 ± 6.27	104.9	11.9
	100	111.9 ± 7.53	111.9	6.73
	200	237.9 ± 19.68	119.0	8.27
sulfathiazole	50	57.96 ± 11.20	115.9	19.3
	100	113.6 ± 6.22	113.6	5.48
	200	208.1 ± 16.91	104.0	8.12
sufameter	50	55.60 ± 8.95	111.2	16.1
	100	124.4 ± 8.48	124.4	6.82
	200	261.9 ± 15.88	130.9	6.06
sulfamethoxy-pyridazine	50	58.90 ± 7.01	117.8	11.9
	100	119.2 ± 6.06	119.2	5.08
	200	247.0 ± 13.29	123.5	5.38
sulfapyridine	50	62.07 ± 5.74	124.1	9.25
	100	128.2 ± 5.11	128.2	3.99
	200	260.8 ± 14.52	130.4	5.57
sulfamethizole	50	53.74 ± 12.8	107.5	23.8
	100	116.9 ± 8.37	116.9	7.16
	200	225.5 ± 9.91	112.8	4.39

sulfamethizole, sulfachlorpyridazine, sulfamerazine, sulfadoxine, sulfadiazine, sulfaquinoxaline, sulfamethazine, sulfamethoxazole, sulfamoxole, sulfadimethoxine, and sulfisoxazole were 0.1, 0.2, 0.4, 1.5, 1.6, 2.1, 3.0, 6.2, 6.8, 7.0, 10, 22, 23, 27, 27, and 80 ng mL^{-1}, respectively.

Milk Sample Analysis. In the course of sulfonamide analysis in milk, a nine-point standard curve was included in each ELISA plate to estimate analyte concentrations. To gain basic information on matrix effect, conformity of standard curves generated in PBS was compared with that of curves obtained using milk matrices. The inhibition curves generated in PBS were consistent with those prepared in 10, 20, or 100 times diluted milk matrices, but the OD value curves differed significantly. After removal of the protein with acetone, the milk samples were diluted 10, 20, or 50 times with PBS, 1% BSA in PBS, 0.5% FG in PBS, or 0.05% Tween 20 in PBS, respectively. The dilution of 20 times with PBS was found to be good to reduce matrix effects for the detection of sulfisozole, sulfathiazole, sufameter, sulfamethoxypyridazine, sulfapyridine, sulfamethizole, and only these six sulfonamides could be detected below MRL after dilution. The standard curves of these six sulfonamides are shown in **Figure 3**, and the standard curves of sulfisozole in PBS or milk samples are shown in **Figure 4**. The results of the recoveries of the six sulfonamides added to milk at 50, 100 (the maximum residue level of sulfonamides in milk), and 200 ng mL^{-1} against a calibration curve set up in buffer are shown in **Table 4**.

The average recoveries ($n = 4$) obtained from the matrices were found in the range of 104−131%. The interassay coefficient of variation (CV) for 100 ng mL^{-1} diluted milk, which

was the concentration of interest, was below 10%; intra-assay variabilities were below 15%. The competitive direct ELISA presented here could be used to determine sulfisozole, sulfathiazole, sufameter, sulfamethoxypyridazine, sulfapyridine, and sulfamethizole in milk simultaneously.

Real milk samples were bought from local markets for sulfonamide screening and determination. Analysis of the 60 food samples using the direct ELISA resulted in 9 samples being positive (sulfonamide concentration was greater than the LOD of the assay). The confirmation step with the HPLC analysis also resulted in detection of the same positive samples, demonstrating that the developed assay can be used for real sample analysis.

Conclusion. This synthetic method of sulfonamide haptens avoided the purification of the intermediate, saved most of the reaction time, and achieved higher yield. It is a good method for the synthesis of haptens that expose the common structures of sulfonamides. The developed ELISA method was capable of detecting 11 of the tested 16 sulfonamides within a concentration range of 1.3−90 ng mL^{-1} in buffer. After the deposition of protein using acetone, milk samples were analyzed by ELISA directly; the matrix effect could be avoided when 1:20 dilution with phosphate-buffered saline was used. The developed immunoassay is suitable to determine sulfisozole, sulfathiazole, sufameter, sulfamethoxypyridazine, sulfapyridine, and sulfamethizole in milk rapidly and reliably.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy spectra; MS, mass spectrometry; TLC, thin-layer chromatography; UV, ultraviolet−visible; MRL, maximum residue limit; LOD, limit of detection; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; TMB, 3,3′,5,5′-tetramethylbenzidine; EDC, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride; IC_{50}, concentration of analyte giving 50% inhibition of color development; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; CB, coating buffer.

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