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Short communication

Organic solvents-free technique for determining sulfadimethoxine and its metabolites in chicken meat

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Abstract

A quick and cost-effective technique of sample preparation followed by a reversed-phase high performance liquid chromatography under "organic solvent-free" (=100% aqueous) conditions for the simultaneous quantifying of sulfadimethoxine (SDM) and its metabolites, 6-hydroxy SDM (6-OH) and N^4-acetyl SDM (N^4-Ac), in chicken muscle is presented. Analysis by HPLC with photo-diode array detector was performed using a short C1 column with an isocratic 0.04 mol/l citric acid mobile phase. The method was validated by the analyses of spiked chicken muscle samples, resulting recoveries (≥84%; relative standard deviations ≤6%), analytical total time (<1/2 h/sample, where a batch of 12 samples in 4 h), and limits of quantitation (≤0.1 μg/g). The decision limits and detection capability were 0.019–0.106 and 0.054–0.112 μg/g, respectively. No organic solvents were used at all.

Keywords: Sulfadimethoxine; Metabolites; Chickens; Organic solvents free

1. Introduction

Sulfadimethoxine (N^1-(2,6-dimethoxy-4-pyrimidinyl) sulfanilamide, SDM), which is a synthetic anti-microbial, normally administered via the food, is applied to prevention of chicken infections with a high frequency. Overuse or misuse of SDM in chickens is of great concern because it can result in its appearance in marketed chicken meat.

A previous author's paper on hepatic biotransformation *in vitro* of SDM in chickens has been found that N^4-acetylation, N^4-acetyl (N^4-Ac) SDM, and hydroxylation in one way, at the 6-position of the pyrimidine ring, 6-hydroxy (6-OH) SDM (Fig. 1), occur; the 6-hydroxyation rate is significantly higher than the N^4-acetylation rate; de-acetylation of N^4-Ac is also detected; consequently, the results suggest a lack of the glucuronidation [1]. The presence of N^4-Ac and 6-OH has been found in chicken excreta *in vivo* [2].

Because determinations for 6-OH and N^4-Ac together with SDM in chicken meats are therefore an important specific activity to guarantee food safety, a useful analytical method for the simultaneous determining SDM, 6-OH, and N^4-Ac is presently required. The acceptable (or ideal) method must have the following qualities: it must be simple, be economical in time and cost, and cause negligible harm to the environment and analyst.

Previous methods for determination of sulfonamides and their metabolites in biological samples including animal tissues and foods of animal origin [3–13] have crucial disadvantages:

(1) All of the methods consume organic solvents as the HPLC mobile phases as well as organic solvents for extraction and deproteinization in sample preparation. Discharging organic solvents is a severe problem on the world-wide scale [10–20]. Risks associated with these solvents extend beyond direct implications for the health of humans and wildlife to affect our environment and the ecosystem in which we all reside. Additionally, incineration for disposal of waste organic solvents has steadily increasing over the past 10 years and has spent huge amounts of money [19,20]. From a standpoint of environment conservation, human health, and economy, reducing (eliminating, if possible) the use of organic solvents is a very important goal.
(2) Most of the methods could not detect simultaneous SDM and its OH/Ac metabolites.

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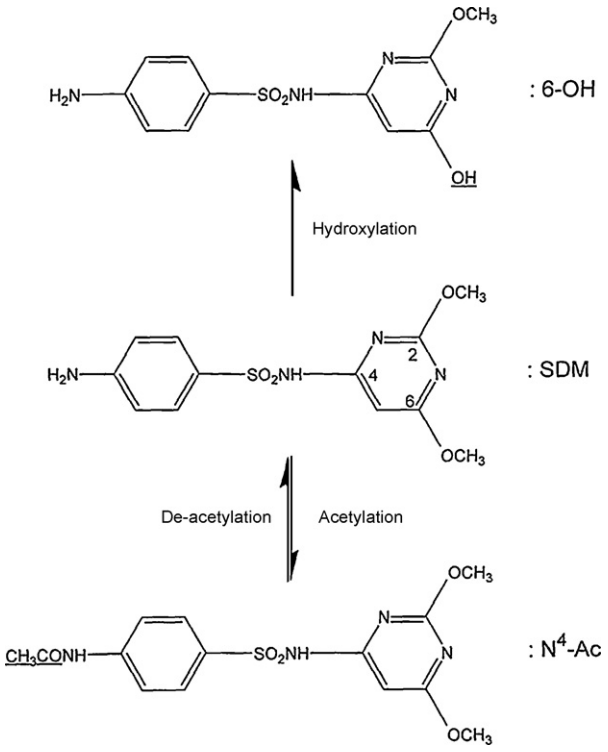

Fig. 1. Structures of biotransformations of SDM.

The organic solvent-free technique described here is a revolutionary method that enables rapid and low-cost determination of SDM, 6-OH, and N^4-Ac in chicken muscles under 100% aqueous conditions.

2. Experimental

2.1. Reagents

Sulfadimethoxine (SDM) standard was purchased from Wako Pure Chem. Ltd. (Osaka, Japan). A 6-hydroxy SDM (6-OH) and N^4-acetyl SDM (N^4-Ac) were generous gifts from Dr. Miura (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). Other chemicals were obtained from Wako. Distilled water was of HPLC grade. A 20% (w/v) perchloric acid (PCA) prepared freshly by diluting refrigerated 100% (w/v) PCA. Stock standard solutions of SDM, 6-OH, and N^4-Ac were prepared by dissolving each of the compounds in water to a concentration of 100 μg/ml. Working mixed standard solutions of these compounds were prepared on by diluting the stock solution with water. These solutions were can be kept in a refrigerator for up to 1 month.

2.2. Apparatus

The following apparatuses were used in the sample preparation: a handheld ultrasonic-homogenizer (model HOM-100, 2 mm I.D. probe, Iwaki Glass Co., Ltd., Funabashi, Japan); a micro-centrifuge (Biofuge® fresco, Kendo Lab. Products, Hanau, Germany); an Ultrafree®-MC/PL (nominal molecular weight limit = 5000 Da, maximum sample size = 0.5 ml, regenerated cellulose ultra-filtration membrane) (Millipore, Bedford, MA) as a centrifugal ultra-filtration unit.

Seven types of C1 non-polar sorbent columns (3 or 5 μm d_P) (length: 50, 75, or 100 mm × 4.6 mm i.d.) with their guard columns (5 mm × 4.6 mm i.d.) for HPLC analysis were used: Column-A, CAPCELL PAK® C1 UG120 (S-5) (50 mm length) (Fine Chemicals Business Dept., Shiseido Co., Ltd., Tokyo, Japan); Column-B, Daisopak® SP-200-3-C1-P (100 mm length) (Daiso Co., Ltd., Osaka, Japan); Column-C, Daisopak® SP-200-3-C1-P (75 mm length) (Daiso); Column-D, Develosil® TMS-5 (50 mm length) (Nomura Chemical Co., Ltd., Aichi, Japan); Column-E, Develosil® TMS-3 (50 mm length) (Nomura Chemical); Column-F, YMC-Pack® TMS (75 mm length) (YMC Co., Kyoto, Japan); Column-G, Wakosil® 5TMS (Wako). Table 1 lists the particle physical specifications.

2.3. HPLC

The HPLC system included a model PU-980 pump and DG-980-50 degasser (both from Jasco Corp., Tokyo, Japan), as well as a model SPD-M10A$_{VP}$ photo-diode array (PDA) detector (Shimadzu Scientific Instruments, Kyoto, Japan). The analytical column was a Daisopak® SP-200-3-C1-P (75 mm × 4.6 mm, 3 μm) column (Daiso) equipped with a guard column (5 mm × 4.6 mm) containing the same packing material. The isocratic mobile phase was a 0.04 mol/l citric acid, and the flow rate was 1.0 ml/min. PDA detector was operated at 190–350 nm: monitoring wavelength was adjusted to 273 nm, which is an average maximum for all the target compounds. The column temperature was operated at 40 °C.

2.4. Sample preparation

An accurately weighed 0.1 g chicken thigh muscle sample homogenized was placed in micro-centrifuge tube and homogenized with a handheld ultrasonic homogenizer for 30 s with 0.8 ml of 20% (w/v) PCA. After being homogenized, the capped tube was centrifuged at 12,000 × g for 5 min. A 50 μl portion of supernatant liquid was placed into an Ultrafree-MC/PL and centrifuged at 5000 × g for 5 min. A 20 μl of the ultra-filtrate was injected into the HPLC system.

3. Results and discussion

3.1. Sample preparation and optimal HPLC condition

Because the high polarity OH metabolites of sulfonamides including SDM [3,8,21,22] are significantly difficult to separate from interferences peaks in the reversed-phase HPLC analysis, the sample preparation required effective/satisfactory purification. The present procedure provided an easy-to-use, rapid, non-use of organic solvents, and shorter operating time, resulting in high recovery and reproducibility with considerable saving of analysis costs.

To optimize the separation with a 100% aqueous mobile phase and provide a more rapid separation, this study tested

Table 1
Physical/chemical specifications of the C1 columns[a] examined and chromatographic analyte peak forms obtained under the HPLC condition ranges examined[b]

Designation	Column length (mm)	d_p (μm)	Surface area (m^2/g)	Pore diameter (Å)	Pore volume (mL/g)	Carbon content (%)	HPLC chromatographic target compounds	
							Separation	Peak form
(A) CAPCELL PAK C1 UG120 (S-5)	50	5	300	120	Nd[c]	4	Not separated[d]	Broadening
(B) Daisopak SP-200-3-C1-P	100	3	300	200	1.1	3	Remarkably	Slight tailing
(C) Daisopak SP-200-3-C1-P	75	3	300	200	1.1	3	Remarkably	Sharp
(D) Develoosil TMS-5	50	5	300	140	1.15	4.5	Not eluted[e]	
(E) Develoosil TMS-3	50	3	300	140	1.15	4.5	Not eluted[e]	
(F) YMC-Pack TMS	75	5	Nd	120	Nd	4	Not separated[d]	Rounded
(G) Wakosil 5TMS	100	5	Nd	120	Nd	4	Not separated[d]	Rounded

[a] i.d. = 4.6 mm.
[b] Mobile phases of molar concentrations ≤ 0.1 mol/l; column temperatures ≥ 25 °C; HPLC flow rates ≥ 0.5 ml/min; HPLC measuring time ≤ 20 min/run.
[c] No data.
[d] Among 6-OH, SDM, N^4-Ac, and the interference of the resulting chicken muscle sample extract.
[e] The column was difficult to elute SDM and N^4-Ac peaks within 20 min (=the HPLC measuring time examined).

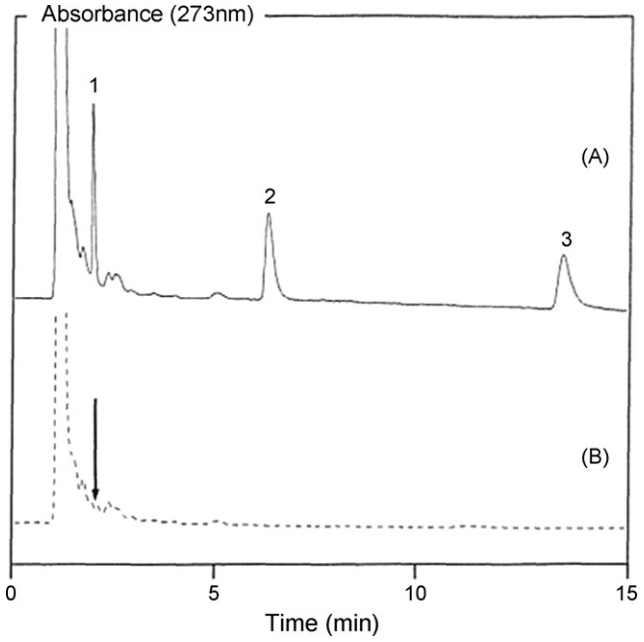

Fig. 2. Chromatograms obtained from the HPLC system. (A) A chicken muscle sample spiked with the target compounds (0.2 μg/g 6-OH, 0.4 μg/g SDM, and 0.4 μg/g N^4-Ac) and (B) a blank chicken muscle sample. Peaks: (1) 6-OH (retention time (Rt) = 1.9 min); (2) SDM (Rt = 6.3 min); (3) N^4-Ac (Rt = 13.7 min). An arrow indicates the retention time of 6-OH.

seven types of the short packed C1 (the highly purified silica-based reversed-phase) columns. The physical/chemical specifications are listed in Table 1. The author used a citric acid solution as the isocratic aqueous mobile phase and examined mobile phases with molarities of citric acid of ≤0.1 mol/l, column temperatures ≥25 °C, HPLC flow rates ≥0.5 ml/min, and HPLC measuring times (retention times) ≤20 min: because the HPLC separations were performed serially, the time per run became critical in routine residue monitoring. The short run time not only increased sample throughput for analysis but also affected the method-development time.

The seven columns were compared with regard to how the analysis separated among 6-OH, SDM, N^4-Ac, and their interfering peaks and the sharpness of the peaks obtained upon injection of equal amounts. The observed chromatographic separations and peak forms formed within the condition ranges examined are also presented in Table 1.

A HPLC chromatogram with complete separation of 6-OH, SDM, N^4-Ac, and interfering peaks, their symmetrical natures, and their short retention times was obtained by the Column-C (Table 1) and an isocratic mobile phase of 0.04 mol/l citric acid with a flow rate of 1.0 ml/min and a column temperature of 40 °C. The HPLC analysis accomplished optimum separation within 14 min and enabled also the multiple sequential injections, without the risks of interfering late-eluting peaks. Under the low-pH environment of the mobile phase and raised column temperature, the short length, 3 μm particle size, and 3% carbon contents in the column were necessary at least to obtain the findings. There was no interference from chicken muscle extract with the elution of 6-OH, SDM, and N^4-Ac, respectively, as illustrated in Fig. 2.

Table 2
Method validation data

	Spiked (μg/g)	6-OH	SDM	N^4-Ac
Linearity (r[a])		0.9996	0.9999	0.9995
Accuracy[b] and precision[c]	0.2	89 (4)	95 (4)	84 (6)
	0.4	87 (5)	92 (4)	86 (5)
CCα[d]		0.019	0.106	0.030
CCβ[e]		0.054	0.112	0.104

[a] r is the correlation coefficient ($P < 0.01$). Mean of three determinations using spiked chicken muscle samples for calibration curves; range of concentration was 0.1–1.0 μg/g.
[b] Data are expressed as average recoveries (%, $n = 5$).
[c] Relative standard deviations in parentheses (%).
[d] Decision limit (μg/g).
[e] Detection capability (μg/g) by the EU regulation 2002/657/EC.

3.2. Method validation

The complete method was qualified in terms of analytical performance parameters. Table 2 summarizes the main performance parameters.

3.2.1. Linearity

The author generated the spiked recovery graph as practical calibration line by plotting peak areas of fortified sample extracts ranging from 0.1 to 1.0 μg/g versus their concentrations. The resulting line showed an excellent linearity for individual compound ($r \geq 0.9995$, $P < 0.01$).

3.2.2. Accuracy and precision

The average recoveries from chicken muscle samples at two different spiking levels (0.2 and 0.4 μg/g) were ≥84%, with relative standard deviations (RSDs) of ≤6%.

3.2.3. Decision limit (CCα) and detection capability (CCβ)

The CCα and CCβ values calculated according to the EU regulation decision (2002/657/EU) [23] are shown in Table 2.

3.2.4. Selectivity

The photo-diode array detector chosen provides an easy way to confirm peak identity and enable the separation and identification of 6-OH, SDM, and N^4-Ac by their retention times and spectra. The author could identify in a sample with retention times and absorption spectra. The 6-OH, SDM, and N^4-Ac peak spectra obtained from sample were practically identical to those of the standards. The present HPLC system made it unnecessary to use very expensive MS to identify the target compounds.

3.2.5. Ruggedness

Some chromatographic parameters were performed using a spiked (0.2 μg/g of each compound) chicken muscle sample obtained under the established procedure.

Changes of ±5% of the HPLC column temperature and the citric acid concentration of the mobile phase had no effect on the peak areas, whereas the variations in the retention times were obtained with the column temperature. Normal retention times for 6-OH, SDM, and N^4-Ac were 1.9, 6.3, and 13.7 min, respectively. By changing the column temperature by +5% (42 °C), the decreasing retention times obtained were 1.5–9.1%, however, no the significant variations were observed with −5% (38 °C). During above studies, all the target compounds were separated.

When the present C1 column was changed after 50 injections of the chicken muscle extract, the new same column contributed no significant difference in the retention times, pear areas, and recoveries of all the target compounds. Changing the guard column also had no effect on the HPLC chromatogram.

4. Conclusions

The proposed technique for determination of SDM its acetyl/hydroxyl metabolites in chicken meat is a useful tool for the routine residue monitoring in chicken and the withdrawal control of chicken farm for the following reasons:

(1) harmless to the environment (does not use organic solvents at all);
(2) shorter analysis time (<1/2 h/sample, where, a batch of 12 samples in 4 h);
(3) economical (budget = €3.63 per sample as at 25 July 2007);
(4) reproducible recoveries (≥84%, with relative standard deviations ≤6%).

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