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Rapid and simple quantitative assay method for diastereomeric flurbiprofen glucuronides in the incubation mixture

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Abstract

Acyl glucuronides of nonsteroidal anti-inflammatory drugs having a chiral center are known to be chemically very active and form covalently bound adducts with proteins, such as human serum albumin, which may be the cause of hypersensitive reactions. Hepatic acyl glucuronosyltransferase catalyzes the transformation of α-aryl propionates into these diastereoisomeric acyl glucuronides, and, hence, its activity needs to be characterized. From this point of view, we developed a rapid, accurate and reproducible analytical method for the separation and determination of diastereoisomeric glucuronides of flurbiprofen, one of the nonsteroidal anti-inflammatory drugs, in the incubation mixture of the hepatic microsomal preparation by high-performance liquid chromatography with a simple column-switching technique for deproteinization. The glucuronides were separated on a TSKgel ODS-80Ts column with 20 m*M* ammonium acetate buffer (pH 5.6)–ethanol–acetonitrile as the mobile phase and monitored with a UV detector at 246 nm. The detection limit of the proposed method was 600 fmol/injection at a signal-to-noise ratio of 10. The validation results indicated that this method would be very useful for the determination of diastereomeric acyl glucuronides formed from flurbiprofen in an incubation mixture.

Keywords: Flurbiprofen glucuronides

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), α-aryl propionic acid derivatives, have an asymmetric center at the α-position of the carboxyl group and are used as the racemate form in the clinical field. NSAIDs absorbed from the intestine undergo transformation into several metabolites by the action of hepatic enzymes. The major metabolic process is known to be the isomerization of the (*R*)-form to its counterpart, the (*S*)-form, [1–3] via the coenzyme A thio-ester as an active intermediate [4]. Glucuronidation, which is one of the major metabolic pathways for drugs and other xenobiotics [5], also takes place through the inherent carboxyl group [6], and the formed acyl glucuronides are excreted into the blood and urine [7]. The hepatic UDP-glucuronosyltransferase is responsible for this metabolism and usually, the glucuronidation is believed to be a very important mechanism for the detoxification process. Meanwhile, the acyl glucuronides are chemically very active and react with amino groups on proteins to produce protein-bound adducts [8–12], which may be related to liver injuries caused by hypersensitive reactions [13,14].

Recent studies have shown that α-aryl propionates

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are stereoselectively metabolized to these acyl glucuronides [15–18]. Hamdoune et al. demonstrated the in vitro acyl glucuronidation of flurbiprofen employing a radiochemical technique with [^{14}C]-uridine-5′-diphosphate glucuronic acid as the substrate [19]. This result indicated the two-fold rapid metabolic rate of (*R*)-flurbiprofen into its glucuronide compared to that of the (*S*)-antipode. To characterize the hepatic enzyme, direct evidence for the formation of these glucuronides as well as the existence of the glucuronides in human body fluids is also needed.

The present study describes the development of a simple, rapid and reproducible assay method for diastereomeric flurbiprofen glucuronides in an incubation mixture using high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Materials

Flurbiprofen was kindly donated by the SS Pharmaceutical Co. (Tokyo, Japan), and each enantiomer was obtained by the diastereomeric salting method using *R*-(+)- and *S*-(−)-methylbenzylamine. The potassium salts of the enantiomeric flurbiprofens were prepared by passing them through the lipophilic ion-exchange gel, carboxymethyl Sephadex LH-20 (K^+ form, 0.89 mequiv./g), which was synthesized from Sephadex LH-20 (Pharmacia, Uppsala, Sweden) in our laboratory [20]. Uridine-5′-diphosphate glucuronic acid (UDPGA) was purchased from Nacalai Tesque (Kyoto, Japan). The amino acid conjugates of flurbiprofen with β-alanine, glycine and taurine were also synthesized in our laboratory. All other chemicals and solvents were analytical-grade and all the glassware used was silanized with trimethylchlorosilane.

2.2. Apparatus

The HPLC analysis was carried out using an LC-10Ai system (Shimadzu, Kyoto) equipped with two LC-10Ai pumps and an SPD-M10AVP diode array detector (246 nm). The separation of the flurbiprofen glucuronides was performed on a TSKgel ODS-80Ts column (5 μm, 2.0 mm I.D.×150 mm, Tosoh, Tokyo) using 20 m*M* ammonium acetate buffer (adjusted to pH 5.6)–acetonitrile–ethanol (20:7:2) as the mobile phase with a flow-rate of 0.2 ml/min. For deproteinization and concentration of the solutes, a Shim-pak MAYI-ODS column, methylcellulose-immobilized ISRP silica gel [21], (5 μm, 4.0 mm I.D.×10 mm, Shimadzu) as a trapping column was used via a column switching valve (Fig. 1). After injection of the sample solution, the column was washed with 100 m*M* ammonium acetate buffer (pH 4.0) for 1 min at a flow-rate of 1 ml/min. The sample on the trapping column was then eluted with an analytical mobile phase and directed to the analytical column. The structural characterization of authentic specimens was carried out with a JMS-LCmate double focusing mass spectrometer (JEOL, Tokyo) at the resolution of 750 or 3000 under the electrospray ionization mode and a Hitachi FT-NMR R-3000 spectrometer at 300 MHz (Hitachi, Tokyo).

2.3. Synthesis of (R)- and (S)-flurubiprofen glucuronides

The synthesis of the (*R*)- and (*S*)-flurbiprofen glucuronide benzyl derivatives was carried out according to a previous report [7], and the obtained reaction products were purified by silica gel column chromatography. The fraction of *n*-hexane–ethyl acetate (10:1, v/v) was dissolved in 2 ml of acetoni-

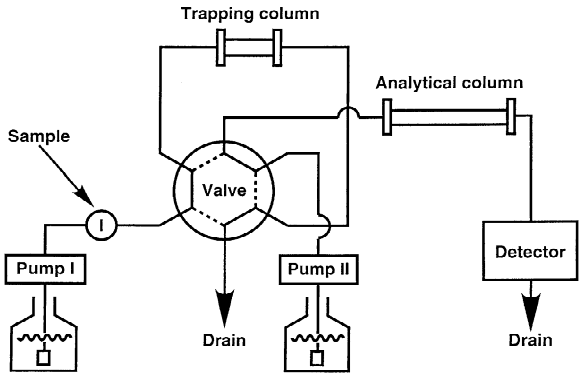

Fig. 1. Schematic diagram of the column-switching system. Trapping column: 5-μm MAYI-ODS column (4.0 mm I.D.×10 mm); analytical column: TSKgel ODS-80Ts (5 μm, 2.0 mm I.D.×150 mm); mobile phase for trapping: 20 m*M* ammonium acetate buffer (pH 4.0) at the flow-rate of 1.0 ml/min; mobile phase for analysis: 20 m*M* ammonium acetate buffer (pH 5.6)–acetonitrile–ethanol (20:7:2, v/v/v) at the flow-rate of 0.2 ml/min; detection: UV 246 nm.

trile, then subjected to preparative HPLC using a Radial-Pak C_{18} cartridge column (10 μm, 8 mm I.D.×100 mm; Waters, Milford, MA, USA) as a separation column for the purification of the corresponding β-anomer. The mobile phases of THF–water (10:9) and acetonitrile–water (3:1) for the *S*-isomer and *R*-isomer, respectively, were used at a flow-rate of 2 ml/min. Benzyl 1-*O*-{(*R*)-2-(2-fluoro-4-biphenylyl)}-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate. ^{1}H NMR ($CDCl_3$) δ: 1.54 (3H, d, J=7.0 Hz, –CH(C$\underline{H}_3$)COOH), 3.56 (1H, t, J=7.8 Hz, 2′-H), 3.67 (1H, t, J=8.8 Hz, 4′-H), 3.80 (2H, m, 2-H and 3′-H), 4.10 (1H, d, J=9.5 Hz, 5′-H), 4.17–5.17 (8H, m, 4×$C_6H_5C\underline{H}_2$–), 5.70 (1H, d, J=7.7 Hz, 1′-H), 6.92–7.39 (28H, m, aromatic H and 4×$C_6\underline{H}_5CH_2$–). HR–MS calc. for $C_{49}H_{45}FO_8Na$ $[M+Na]^+$ 803.2996; found, 803.2988. Benzyl 1-*O*-{(*S*)-2-(2-fluoro-4-biphenylyl)}-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate. ^{1}H NMR ($CDCl_3$) δ: 1.54 (3H, d, J=7.3 Hz, –CH(C$\underline{H}_3$)COOH), 3.61 (1H, t, J=8.4 Hz, 2′-H), 3.67–3.75 (2H, m, 2-H and 4′-H), 3.84 (1H, t, J=8.8 Hz, 3′-H), 4.08 (1H, d, J=9.5 Hz, 5′-H), 4.42–5.13 (8H, m, 4×$C_6H_5C\underline{H}_2$–), 5.70 (1H, d, J=7.7 Hz, 1′-H), 7.08–7.47 (28H, m, aromatic H and 4×$C_6\underline{H}_5CH_2$–). HR–MS calc. for $C_{49}H_{45}FO_8Na$ $[M+Na]^+$ 803.2996; found, 803.2965.

The (*R*)- and (*S*)-flurbiprofen glucuronide benzyl derivatives were dissolved in 0.4 ml of 10 m*M* acetic acid in ethyl acetate and stirred with 18.8 mg (*S*-diastereoisomer) of $Pd(OH)_2$/Pd on carbon (two times the weight of the benzyl derivative) at room temperature for 40 min under a hydrogen gas atmosphere at a pressure of 5 kg/cm^2. After removal of the catalyst by centrifugation at 3000 rpm for 10 min, the supernatant was evaporated in vacuo and the obtained glucuronides were precipitated from ether–*n*-hexane. 1-*O*-{(*R*)-2-(2-fluoro-4-biphenylyl)}-β-D-glucopyranuronic acid [11% yield from (*R*)-flurbiprofen]. HR–MS calc. for $C_{21}H_{20}FO_8$ $[M-H]^-$ 419.1142; found, 419.1175. 1-*O*-{(*S*)-2-(2-fluoro-4-biphenylyl)}-β-D-glucopyranuronic acid [12% yield from (*S*)-flurbiprofen]. HR–MS calc. for $C_{21}H_{20}FO_8$ $[M-H]^-$ 419.1142; found, 419.1145.

2.4. Sample preparation

Standard stock solutions of the (*R*)- and (*S*)-flurbiprofen glucuronides were prepared in methanol at 29.4, 14.7, 7.35, 2.94, 0.735, 0.294 and 0.074 μg/ml, and then stored at −20 °C. These solutions were used for the calibration, accuracy and precision studies. In the calibration study, a 100-μl aliquot of each stock solution was mixed with 400 μl of 100 m*M* sodium phosphate buffer (pH 6.8) and 100 μl of 10% trichloroacetic acid. To the mixtures, 100 μl of a methanolic solution of (*S*)-flurbiprofen–β-alanine conjugate (0.71 μg/ml), as the internal standard, and 50 μl of methanol were then added. A 90-μl aliquot of these mixtures was injected into the analytical system.

For the accuracy and precision studies, a 100-μl aliquot each of the 29.4, 2.94 and 0.074 μg/ml standard stock solutions was evaporated under a nitrogen gas stream at room temperature, followed by dissolution with 100 μl of 10% trichloroacetic acid. This solution was added to 100 m*M* sodium phosphate buffer (pH 6.8, 300 μl) containing 2.5 μmol D-saccharic acid 1,4-lactone, 5 μmol $MgCl_2$, Brij 58 (40 μg) and a male Wistar rat liver microsomal preparation (200 μg of protein). To this mixture, 100 μl of 20 m*M* uridine-5′-diphosphate glucuronic acid, 100 μl of an internal standard stock solution and 150 μl of methanol was further added. The entire solution was mixed well and centrifuged at 3000 rpm for 10 min, and a 90-μl aliquot of the solution was injected into the HPLC system.

2.5. Accuracy and precision of the assay

Analytical errors were investigated using the above samples. In order to evaluate the intra-day validation, six samples each of three different concentrations were prepared on the same day and analyzed. The inter-day validation was evaluated for 6 days. The relative error (RE, %) was calculated as [(found concentration) − (theoretical concentration)/added concentration] × 100 (%), and the precision was obtained in terms of the coefficient of variation (RSD, %).

3. Results and discussion

The synthesis of the diastereomeric acyl glucuronides of flurbiprofen was undertaken by condensation of the (*R*)- and (*S*)-isomers with benzyl-2,3,4-tri-*O*-benzyl-D-glucuronoate through these carboxyl

groups employing the Mitsunobu reaction. The reaction condition, $Pd(OH)_2/Pd$ on carbon instead of Pd on carbon, and the purification procedure were slightly modified from the previously reported method [7]. The ^1H NMR spectrum of both the (*R*)- and (*S*)-flurbiprofen glucuronide benzyl derivatives displayed the characteristic anomeric proton signal at 5.70 ppm as doublets with $J=7.7$ Hz, indicating a β-glucuronosyl linkage. In addition, the high resolution mass spectrometric analysis also characterized the molecular formulas. The ESI-mass spectrum of (*R*)-flurbiprofen glucuronide, which was synthesized by catalytic hydrogenation, clearly supported the reductive removal of the benzyl groups as depicted in Fig. 2. The glucuronide showed a deprotonated molecule $[M-H]^-$ at m/z 419 as a base peak.

It is well known that pH of the mobile phase influences the elution behavior of an ionic compound [22]. Hence, the chromatographic behaviors of diasereomeric flurbiprofen glucuronides on an ODS column were investigated to obtain the best separation conditions. Due to the presence of the diastereomeric isomers, a reversed-phase column seemed to be useful for the liquid chromatographic resolution of the (*R*)- and (*S*)-flurbiprofen glucuronides in biological fluids. The k'-values of the glucuronides relative to taurine-conjugated flurbiprofen, having a sulfonyl group, with a mixed solution of 20 m*M* sodium phosphate buffer adjusted to appropriate pH and acetonitrile as the mobile phase were plotted versus the pH, and the obtained result is depicted in Fig. 3. The relative k'-values of the glucuronides were influenced by the pH of the mobile phase due to the pK_a values (3.0–3.5) of a carboxyl group on the glucuronic acid moiety. It is very interesting that the elution order of the diastereomeric flurbiprofen glucuronides varies at around pH 4. The (*S*)-antipode was more strongly retained on the stationary phase than its counterpart in the acidic region, where the carboxyl group existed in the undissociated form. The same phenomenon has been previously reported with diastereomeric ibuprofen glucuronide [7], and may suggest that the lipophilicity of the (*R*)- and (*S*)-flurbiprofen glucuronides may reverse due to the conformational change caused by the dissociation of the carboxyl group on the sugar moiety. In the pH region of 5–7, both glucuronides showed almost the same retention behavior with fine base-line separation. In the higher pH region, the racemic flurbiprofen as a substrate interfered with the resolution of these glucuronides. The use of 20 m*M* ammonium acetate buffer (pH 5.6)–acetonitrile–ethanol (20:7:2) as the mobile phase was the most preferable. The glycine, taurine and β-alanine conjugates of flurbiprofen were syn-

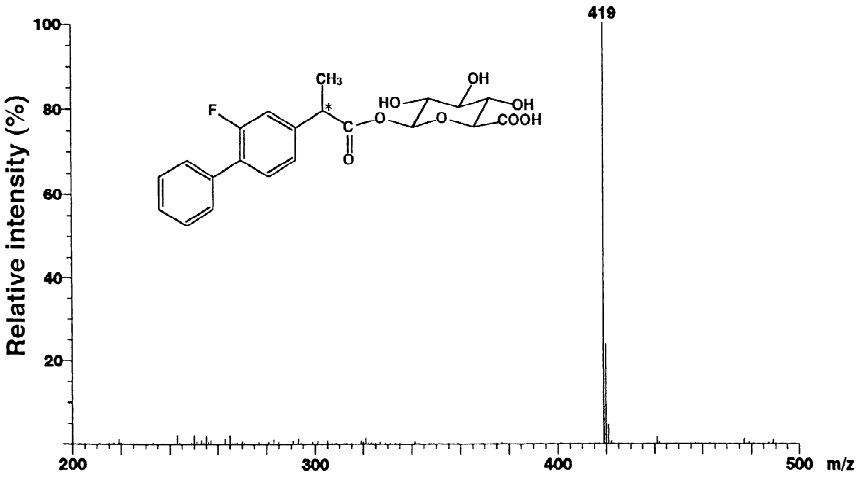

Fig. 2. Electrospray negative ion mass spectrum of (*R*)-flurbiprofen glucuronide. Conditions: mobile phase: water–methanol (1:1, v/v); flow-rate: 0.2 ml/min; injection amount: 3 ng; electrospray voltage: −2.5 kV; orifice voltage: −20 V; ring lens voltage: −100 V; orifice temperature: 150 °C; desolvating plate temperature: 250 °C.

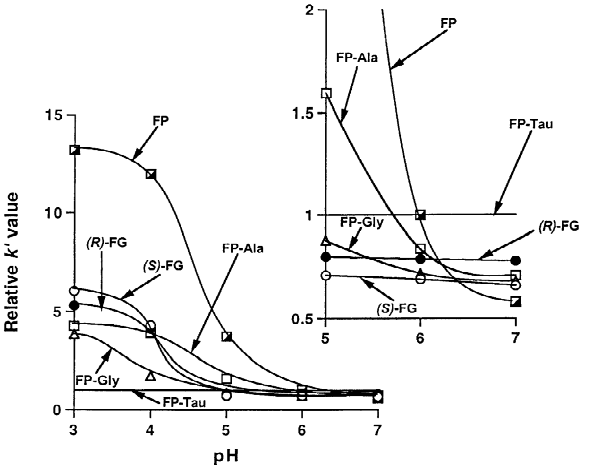

Fig. 3. Effect of the pH of a mobile phase on k'-values relative to taurine-conjugated (*S*)-flurbiprofen. FP = Flurbiprofen, FG = flurbiprofen glucuronide, FP-Ala = (*S*)-flurbiprofen–β-alanine conjugate, FP-Gly = (*S*)-flurbiprofen glycine conjugate, FP-Tau = (*S*)-flurbiprofen–taurine conjugate. Chromatographic conditions: mobile phase: 20 m*M* sodium phosphate buffer–acetonitrile (2:1, v/v, for pH 3 and 4; 5:2, v/v, for pH 5–7); column: TSKgel ODS-80Ts (5 μm, 2.0 mm I.D.×150 mm); flow-rate: 0.2 ml/min; detection: UV 246 nm; injection amount: 7 ng for (*S*)-flurbiprofen, 4 ng for (*S*)-flurbiprofen–glycine conjugate, 6 ng for (*S*)-flurbiprofen–β-alanine conjugate, (*R*)- and (*S*)-flurbiprofen glucuronides.

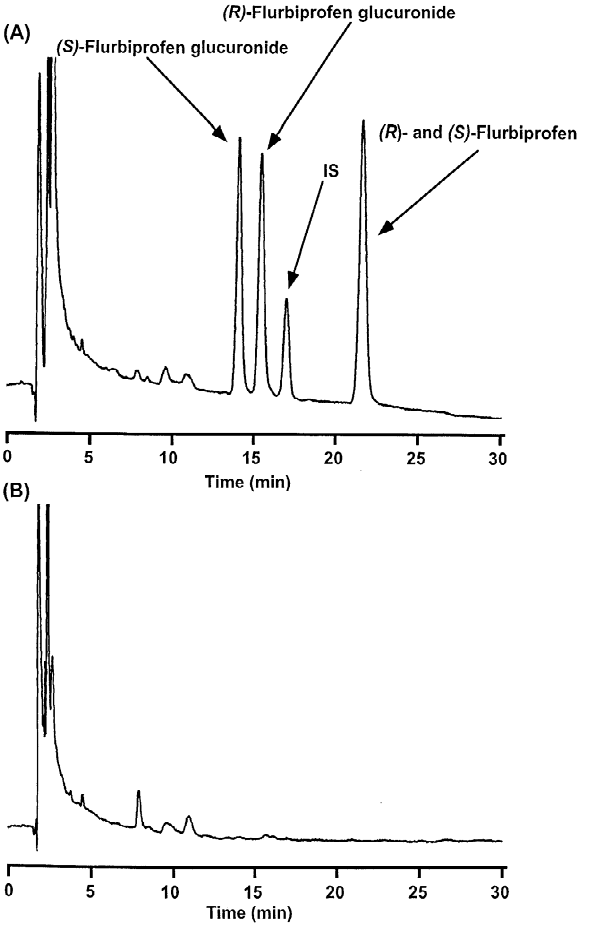

Fig. 4. Typical chromatogram of the incubation mixture spiked with (A) and without (B) standard target compounds analyzed by the column-switching system. Chromatographic conditions were the same as in Fig. 3.

thesized and their chromatographic behaviors were investigated. These results indicated the β-alanine conjugate as the most desirable internal standard for the assay system (Fig. 3).

Due to the instability of the target compounds, a common extraction procedure, solid-phase extraction with an ODS silica gel cartridge or reversed-phase polymer cartridge, was not effective for the quantitative extraction of the flurbiprofen glucuronides produced in the incubation mixture. Therefore, a simple column-switching system attached to a trapping column, a MAYI-ODS column to remove hydrophilic biopolymers such as proteins [21], was developed as illustrated in Fig. 1. Adding trichloroacetic acid prior to the injection was also combined for denaturation of the microsomal proteins. The typical chromatogram obtained by injection of an incubation mixture spiked with the standard flurbiprofen glucuronides (each 34.8 ng/injection), β-alanine conjugate (34.8 ng/injection) and substrate (8.7 ng/injection) is shown in Fig. 4, where well resolved peaks of the target compounds were observed without any interference. The detection limit was 600 fmol/injection of the flufbiprofen glucuronides at a signal-to-noise ratio of 10.

Next, we investigated the validation of this assay method. The samples for calibration were analyzed three times and the results are shown in Table 1. Excellent correlation coefficients (more than 0.999) were obtained for both the (*R*)- and (*S*)-flurbiprofen glucuronides with a dynamic range of 400-fold (7.3 ng to 2.94 μg/sample). The results of the intra-day ($N = 6$) and inter-day ($N = 6$) accuracy and precision are also shown in Table 2. These results indicate that

Table 1
The precision and accuracy in analysis of calibration samples ($N=3$)

	Concentration (μg/ml)						
	0.074	0.29	0.74	2.9	7.4	14.7	29.4
(R)-Flurbiprofen glucuronide							
Found	0.0733	0.319	0.708	2.87	7.08	14.78	30.97
RSD (%)	5.6	6.7	1.4	3.4	5.0	2.8	1.5
RE (%)	−1.0	8.6	−3.7	−2.5	−3.6	0.9	5.4
(S)-Flurbiprofen glucuronide							
Found	0.0768	0.259	0.741	3.03	7.55	15.45	31.34
RSD (%)	26.9	7.2	1.0	5.7	1.2	4.9	2.6
RE (%)	3.7	−11.8	0.8	3.0	2.7	5.5	6.7

the proposed method is satisfactory for the determination of the diastereomeric flurboprofen glucuronides in an incubation mixture with high accuracy and precision.

Recent papers have shown that the acyl glucuronide of a drug produces covalently bound adducts with proteins [8–12], which may be responsible for the hypersensitivity reactions of acidic compounds [13,14]. It is also reported that the depression of producing protein-bound adducts by adding inhibitors of the acyl glucuronosyltransferase is also effective for reducing the toxicity [23]. In addition, we have recently found that rat hepatic bile acid acyl glucuronosyltransferase is effectively inhibited by the amino acid conjugates of bile acids [24]. The developed method would be very useful for the characterization of hepatic acyl glucuronosyltransferase for acidic drugs and elucidation of the inhibitory mechanism of the enzyme activity.

Table 2
Intra- and inter-day validation in analysis of (*R*)- and (*S*)-flurbiprofen glucuronides in the incubation mixture

	Standard added (μg/ml)		
	0.074	2.9	29.4
(R)-Flurbiprofen glucuronide			
Intra-day ($N=6$)			
Found	0.0755	2.78	29.25
RSD (%)	1.84	2.89	1.61
RE (%)	1.99	−5.42	−0.52
Inter-day ($N=6$)			
Found	0.0778	2.79	29.04
RSD (%)	6.51	3.17	3.55
RE (%)	5.13	−4.97	−1.23
(S)-Flurbiprofen glucuronide			
Intra-day ($N=6$)			
Found	0.0773	2.90	29.78
RSD (%)	5.64	2.33	4.05
RE (%)	4.43	−1.46	1.30
Inter-day ($N=6$)			
Found	0.0768	2.85	30.19
RSD (%)	9.59	3.15	2.26
RE (%)	3.83	−3.03	2.70

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