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Note

Determination of a new hypolipidemic agent, 2-(*trans*-4-isobutylcyclohexyl)-2-oxoethyl benzenesulphonate, and its metabolite by high-performance liquid chromatography and gas chromatography–mass fragmentography in human biological materials

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2-(*trans*-4-Isobutylcyclohexyl)-2-oxoethyl benzenesulphonate (I, FL-386) is a new derivative of benzenesulphonic acid [1,2]. It has a very low toxicity [3], and was developed as a lipase inhibitor and hypolipidemic agent to decrease the risk of atherosclerosis.

As shown in Fig. 1, compound I is metabolized in the body mainly to benzene-

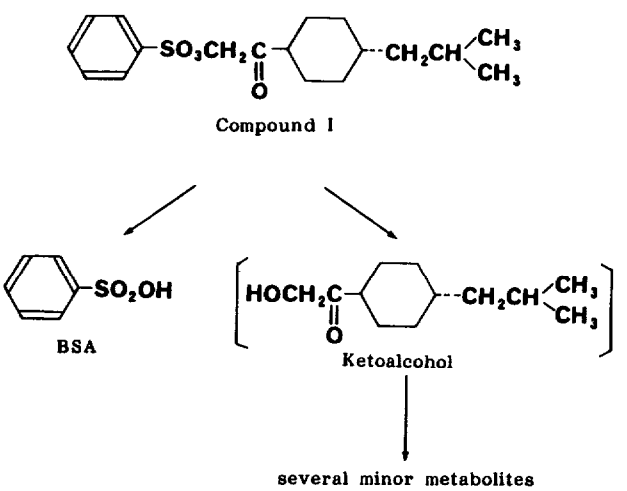

Fig. 1. Metabolic pathway of compound I.

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sulphonic acid (BSA) and various minor metabolites through 2-(*trans*-4-isobutylcyclohexyl)-2-oxoethanol (ketoalcohol) as an intermediate. The chemical structures of these minor metabolites could not be assigned, since they were present in only trace amounts.

We have examined the determination of I in human biological materials by high-performance liquid chromatography (HPLC) on a reversed-phase column with UV detection, and that of BSA as its ethyl derivative by gas chromatography–mass fragmentography (GC–MF) on a capillary column.

EXPERIMENTAL

Materials and reagents

Compound I was supplied by our Synthetic Laboratory [1,2]. The metabolite BSA, *p*-toluenesulphonic acid (TSA, used as the internal standard), methanol and *n*-hexane of liquid chromatographic reagent grade, and other chemicals used were all purchased from Wako (Osaka, Japan). A Sep-Pak C_{18} cartridge containing μBondapak C_{18}/Porasil (R/B) used for the extraction was purchased from Waters Assoc. (Milford, MA, U.S.A.). This cartridge was activated with consecutive 5-ml volumes of distilled water, methanol and distilled water before use.

Preparation of samples

Blood samples were collected in heparinized containers and immediately centrifuged (2000 *g*) at 5°C for 15 min to separate the plasma. The plasma, urine and faeces samples were then frozen at −20°C until analysis.

The plasma (1.0 ml) was diluted with 0.5 ml of 1 *M* hydrochloric acid containing 1.0 μg of TSA as an internal standard. This diluted plasma was extracted with 5 ml of *n*-hexane at room temperature for 10 min and centrifuged (2000 *g*, 5°C, 5 min). This extraction was repeated with 3 ml of *n*-hexane, and the combined *n*-hexane extracts containing compound I were evaporated to dryness at water temperature (<20°C), transferred to a 10-ml test tube by washing with *n*-hexane, and dried under nitrogen gas at water temperature. The residue was redissolved in 0.2 ml of methanol, and 20 μl of this solution were injected into the liquid chromatograph.

The partial aqueous layer (1.0 ml), containing the metabolite BSA (separated from the *n*-hexane layer), was applied to a Sep-Pak C_{18} cartridge, washed with 0.5 ml of distilled water and eluted with 4 ml methanol. The methanol eluate was reduced to dryness under a stream of nitrogen gas and redissolved in 0.1 ml of methanol. After refrigerated centrifugation at 2000 *g* for 5 min, the supernatant was ethylated at room temperature overnight by addition of 0.5 ml of diethyl ether solution containing saturated diazoethane. To the supernatant obtained after centrifugation (2000 *g*, 5°C, 5 min), 1.0 ml of distilled water and 10 μl of 1 *M* hydrochloric acid were added, and the solution was extracted with 3 ml of *n*-hexane for 10 min. The *n*-hexane layer was concentrated to a volume of ca. 50–100 μl, and 1 μl of this concentrate was analysed by the GC–MF method.

Whole faeces were homogenized in an ice-bath with 1–2 l of ice-cold physiological saline and centrifuged at 2000 *g* and 5°C for 15 min. The resulting superna-

tant of the faeces homogenates (1.0 ml) or centrifuged urine (1.0 or 0.1 ml) was treated by the same procedure, except that the aqueous layer was applied to a Sep-Pak C_{18} cartridge and eluted with methanol without washing with distilled water; the amount of internal standard TSA added was 5 μg for urine and 1 μg for the faeces homogenates.

High-performance liquid chromatography

A Shimadzu Model LC-6 high-performance liquid chromatograph equipped with a Model SPD-6AV variable-wavelength detector, a Model C-R3A Chromatopac data system and a Model SIL-6A automatic injector (Kyoto, Japan) was used.

A Shimadzu Shim-pack CLC ODS chromatographic column (150 mm×6 mm I.D., 5 μm particle size) was used for the separation. The mobile phase was methanol–distilled water (75:25); the flow-rate was 1.5 ml/min. The column was maintained at 30°C, and compound I eluted was recorded by the detector at a constant wavelength of 220 nm; the attenuator was set at 0.02 a.u.f.s.

Gas chromatography–mass fragmentography

A JEOL Model JMS DX-303 gas chromatograph–mass spectrometer, with an electron-impact (EI) ion source and equipped with a Model JMA-DA5100 data system (Tokyo, Japan), was used.

The chemically bonded fused-silica capillary column of the gas chomatograph was coated with methylsilicone (25 m×0.33 mm I.D., Shimadzu) and was conditioned at 280°C for 12 h. The injector, separator and ion source temperatures were 200, 250 and 180°C, respectively. Analyses were carried out with an initial temperature of 70°C and a temperature rise of 16°C/min to 250°C. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The split ratio was 20:1. The splitless injection was carried out using a moving needle.

The mass spectrometer was operated under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3.0 kV; ion multiplier voltage, 1.2–1.4 kV. The mass fragment ions selected were as follows: BSA, the *m/z* 186 ion for all samples; the internal standard TSA, the *m/z* 200 ion for the plasma and urine samples and the *m/z* 155 ion for the faeces sample.

Calibration curves

A series of standard solutions of compound I containing 0.1, 0.5, 1.0, 5.0 and 10.0 μg/0.2 ml for all biological samples in 0.2 ml of methanol was prepared. A 20-μl sample of each standard solution was analyzed by the present HPLC method, and a calibration curve was obtained by plotting the peak area of I against concentration. A linear calibration curve was obtained ($y=8566.869x-444.0063$, $r=0.9999242$).

A calibration curve was prepared by adding known amounts of BSA (1, 5, 10, 50 and 100 ng/ml) and the internal standard TSA (1 μg for plasma and faeces, 5 μg for urine) to 1.0-ml aliquots of distilled water, and then analysing the mixture by GC–MF using the same preparation procedure without extraction with *n*-hexane; the ratio of the peak height of BSA to that of TSA was plotted against the concentration of I. Each calibration curve was linear: $y=0.001071189x-$

0.01488459 ($r=0.9998738$) for plasma, $y=0.00002218074x-0.0489865$ ($r=0.9937758$) for urine and $y=0.0003278765x-0.007093013$ ($r=0.9999195$) for faeces.

RESULTS AND DISCUSSION

Of the extraction procedures tested for human biological materials, the following procedures were found to be the most reliable with the highest recovery for compound I and BSA, and no decomposition was observed. Extraction with *n*-hexane from an aqueous solution, adjusted to pH 3.0 with 1 *M* hydrochloric acid, was first employed for quantitative separation of I from BSA. Compound I in the *n*-hexane extract was then analysed by HPLC on a reversed-phase column. GC or GC–MF was not suitable for the assay of I because of its decomposition in the heated injection port.

Subsequently, the preparation with a Sep-Pak C_{18} cartridge was used to extract BSA from the aqueous layer separated after extraction with *n*-hexane. The aqueous layer containing the remaining BSA was applied to the activated cartridge and, except for the urine and faeces homogenates, washed with distilled water and subsequently eluted with methanol. The dried residue of the methanol eluate was ethylated and subsequent extracted with *n*-hexane. This procedure resulted in no interfering peaks from biological constituents following chromatography by GC–MF on a capillary column, whereas methylation did not give good separation. Neither the procedure without extraction with *n*-hexane after ethylation on the GC–MF analysis nor reversed-phase HPLC could be employed because of poor separation from the biological components. There have been no reports of the determination of BSA in biological materials.

Compound I was well separated from biological constituents when most reversed-phase chromatographic columns were used. After various tests, a Shimpack CLC ODS column was chosen for the separation, and isocratic elution with methanol–distilled water (75:25) was used, with UV detection at 220 nm.

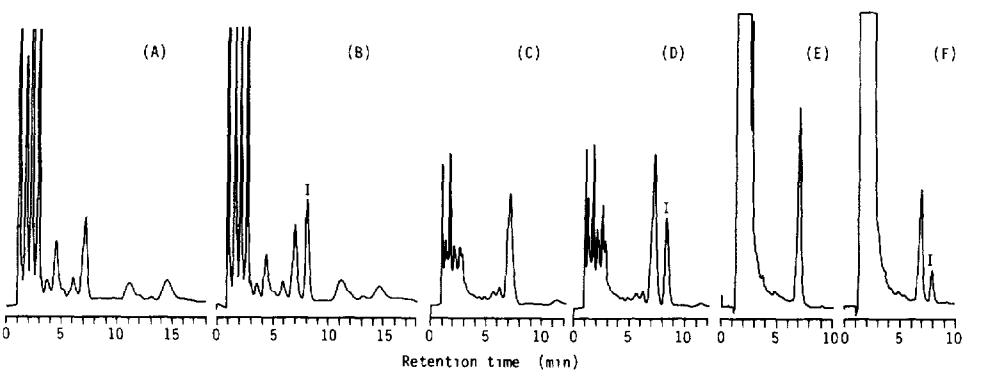

Fig 2. Typical liquid chromatograms showing the separation of blank human (A) plasma, (C) urine and (E) faeces, compound I in human (B) plasma and (D) urine (each 1.0 μg/ml) prepared following addition and in (F) faeces homogenates (ca. 0.5 μg/ml) prepared after administration. Details of the HPLC conditions are in the text.

Typical chromatograms showing the separation of blank, compound I prepared from human plasma and urine following addition of I (1 μg/ml) and faeces homogenates after administration of I (200 mg) are shown in Fig. 2. The retention time of I, under HPLC conditions described, was ca. 8 min. The degree of chromatographic interference from constituents endogenous to all biological materials was negligible.

The internal standard, TSA, for the GC–MF analysis of BSA was chosen by the multiple-ion detection technique, because it showed a similar EI mass spectral pattern to that of BSA, the same recovery from biological materials and a suitable retention time on GC–MF analysis.

The following molecular ion peaks and characteristic fragment ion were selected for the GC–MF analysis: the *m/z* 186 ion $[M]^{+\cdot}$ of BSA for all samples, the *m/z* 200 ion $[M]^{+}$ of TSA for plasma and urine samples and the *m/z* 155 ion $[M-OC_2H_5]^{+}$ of TSA for faeces sample. None of these fragment ions detected had any appreciable influence on the biological constituents and gave an excellent

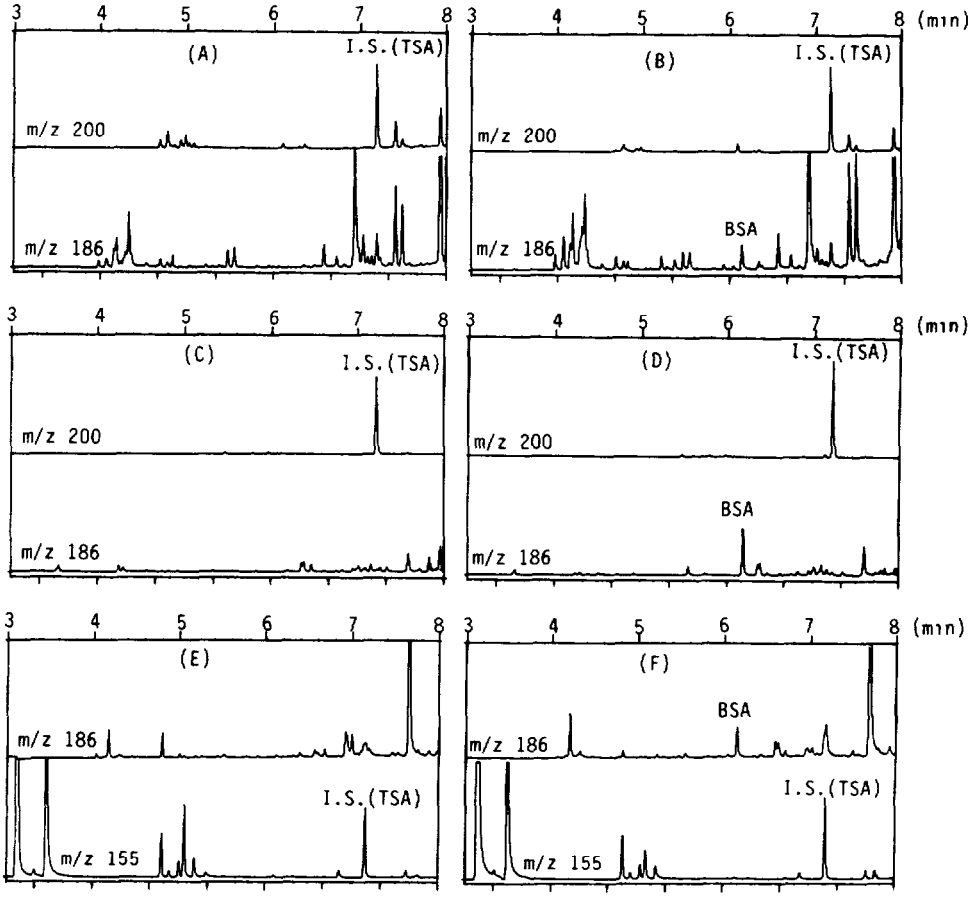

Fig 3. Typical mass fragmentograms showing the separation of blank human (A) plasma, (C) urine and (E) faeces, and metabolite BSA and internal standard (I.S., TSA) prepared from human (B) plasma, (D) urine and (F) faeces. Details of the GC–MF conditions are in the text.

separation on measurement by GC–MF. The $[M]^+$ ion of TSA for faeces sample was not separated clearly in some samples.

Typical GC–MF chromatograms of BSA and TSA prepared from human plasma, urine and faeces following administration of compound I are illustrated in Fig. 3. The retention times of BSA and TSA were ca. 6.2 and 7.2 min, respectively.

Known amounts of compound I and its metabolite BSA were added to blank plasma, urine and faeces homogenates of human at concentrations of 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml for I and 1, 5, 10, 50 and 100 ng/ml for BSA, respectively, and the samples were analysed by HPLC and GC–MF. The recovery ($n=3$ for each added amount, mean ± S.D.) of each compound prepared from plasma was 96.2 ± 5.3% for I and 74.9 ± 4.0% for BSA; that from urine was 94.5 ± 6.9% for I and 77.8 ± 7.9% for BSA; and that from faeces homogenates was 91.7 ± 3.9% for I and 78.5 ± 2.3% for BSA.

The detection limit of I using the HPLC method was 0.1 μg/ml of all samples and that of BSA by the GC–MF method was 1 ng/ml in plasma and urine or 5 ng/ml in faeces homogenates; the reproducibility was ± 2.1–3.3% and ± 2.5–4.2%.

Although the results of the clinical pharmacological studies on compound I will be reported elsewhere, active substance I was observed only in faeces, not in any samples of plasma or urine. Inactive substance BSA was detected in all samples, whereas other metabolites could not be detected. Compound I may possibly not be absorbed in an unchanged form as well as other hypolipidemic agents, since it was activated in the alimentary canal. Therefore, compound I could not be detected in plasma and urine. For the pharmacological studies of I, it may be necessary to use mainly the pharmacodynamic results for BSA, because compound I may not be monitored in most biological materials.

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