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High-performance liquid chromatographic determination of sulphobromophthalein and its conjugates

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ABSTRACT

A simple, sensitive and selective high-performance liquid chromatographic method for the determination of sulphobromophthalein and its mercaptide conjugates in rat bile was developed. These pigments, which have an absorption maximum at 580 nm in alkaline solution, were separated isocratically on an alkali-resistant ODS column by paired-ion chromatography. Analysis of bile samples obtained after intravenous administration of sulphobromophthalein to rats showed the presence of at least twenty peaks of metabolites, of which thirteen were identified and seven quantified.

INTRODUCTION

Sulphobromophthalein sodium (BSP) has been used in the clinical evaluation of hepatic dysfunction and as a useful tool for the investigation of hapatobiliary transport mechanisms of drugs. Despite many publications on the metabolism of the dye, however, only four conjugates have been reported as its metabolites in several animals [1,2]: BSP–monoglutathione (BSP–mGSH) as the major metabolite, BSP–diglutathione (BSP–dGSH), BSP–monocysteinylglycine (BSP-mCys-Gly) and BSP-monocysteine (BSP–mCys) as the minor metabolites. In most of these studies, paper chromatography (PC), paper electrophoresis and thin-layer chromatography (TLC) have been used as the major tools for the separation [3–6]. These methods require considerable time for development, and the resolution, sometimes, lacks precision.

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Since *in vivo* and *in vitro* formation of BSP–dGSH has been reported [2,7], it is reasonable that the diconjugate may be synthesized through intermediate formation of two positional isomers of BSP–mGSH. Moreover, it has been found that GSH itself transported by hepatocytes into bile canaliculi is hydrolysed into Cys-Gly, Cys and γ-glutamylcysteine (Glu-Cys) by sequential action of γ-glutamyltransferase (γ-GTP) and dipeptidase(s) located on the biliary ductular epithelium [8–11]. These studies also suggest that many other BSP–mercaptide conjugates can be found in the bile of animals that have received BSP.

Thus, it was of interest to re-examine the metabolic transformation of BSP and its conjugates. For this purpose, we developed a new high-performance liquid chromatographic (HPLC) method for the determination of BSP–mercaptide conjugates. Separation of their pigments is based on paired-ion chromatography using triethylamine phosphate (TEA–H_3PO_4) as a pairing agent. Since it is predicted that all BSP–mercaptides will show an absorption maximum at 580 nm and

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identical molar absorptivities in alkaline solution, we selected the wavelength in order to detect them selectively and sensitively. The separation was achieved on alkali-resistant ODS column (Capcell Pak C_{18}) using two isocratic mobile phases consisting of 0.1 *M* TEA–H_3PO_4 buffer (pH 9.9) and acetonitrile.

EXPERIMENTAL

Chemicals

BSP was obtained from Aldrich (Milwaukee, WI, USA); GSH and Glu-Cys were from Kozin (Tokyo, Japan); Cys-Gly was from Bachem Feinchem (Bubendorf, Switzerland); cysteine (Cys), N-acetylcysteine and triethylamine were from Kanto (Tokyo, Japan); γ-GTP was from Sigma (St. Louis, MO, USA). Authentic BSP–mercaptide conjugates were prepared from BSP and the respective mercaptides, such as GSH, Glu-Cys and Cys, by a modification of the method of Whelan *et al.* [7] and purified by our new procedure (will be published elsewhere). Briefly, BSP and an individual mercaptide (0.5 mmol each for the preparation of the monomercaptide; 0.5 and 2 mmol for the preparation of the dimercaptide, respectively) were dissolved in 10 ml of 28% ammonium hydroxide and incubated at 37°C for 2 h. The excess ammonia in the reaction mixture was distilled off *in vacuo*. The yellowish syrupy residue, containing BSP–dimercaptide and two isomers of BSP–monomercaptide, was applied to a column of XAD-4 (71–105 μm particle size, 300 mm × 25 mm I.D.) equilibrated with 0.1 *M* triethylamine bicarbonate buffer (pH 9.0). Each pigment was eluted stepwise with 200 ml each of a mixture of the same buffer and methanol (0–40%, v/v). The TEA salt of each BSP–mercaptide was converted into the corresponding sodium salt with Dowex 50W-X8 (H^+ form) resin and IRC-150 (Na^+ form) resin.

HPLC column packing

Capcell Pak C_{18} SG 120 (5 μm, Shiseido, Tokyo, Japan), 3.1 g, was suspended in 20 ml of chloroform and sonicated for 3 min. The packing slurry was then poured into a 15 cm × 0.6 cm I.D. stainless-steel column with a 50-ml reservoir, and the reservoir was filled with a pressuring liquid, methanol–water (1:1, v/v). The packing material was then packed at a constant pressure of 300 bar using the pressuring liquid.

A stainless-steel pre-column (5 cm × 0.46 cm I.D.) was packed with 0.6 g of the same gel suspended in 10 ml of chloroform by the same procedure.

The Capcell Pak C_{18} is prepared by chemical coating of silica gel with a silicon polymer to which octadecyl groups are chemically bonded via Si–C bonds. The silicon coating layer provides excellent stability against alkaline solvents.

HPLC apparatus and chromatography

A Nippon Bunko 880-PU high-performance liquid chromatograph, a variable-wavelength detector set at 580 nm and a Shimadzu CR-4A electronic integrator were used. Two isocratic mobile phases (solvents A and B) were employed. Solvent A, consisting of 0.1 *M* TEA–H_3PO_4 buffer (pH 9.9, at 22.0°C)–acetonitrile (90:13, v/v), was used for the separation of BSP–mercaptide conjugates. For the analysis of unchanged BSP, solvent B, consisting of 0.1 *M* TEA–H_3PO_4 buffer (pH 9.9, at 22.0°C)–acetonitrile (73:27, v/v), was employed. After the elution of BSP–mercaptide conjugates, the mobile phase was changed to solvent B for the separation of BSP. The flow-rate was 1.0 ml/min, and all separations were carried out at room temperature (22–23°C).

Animal experiments and analytical procedure for bile samples

Male Wistar rats (eight weeks old) weighing 230–250 g were anesthetized with sodium pentobarbital, 50 mg/kg intraperitoneally. Through an abdominal incision, the bile duct was cannulated with polyethylene tubing. Body temperature was maintained at 38 ±0.5°C with a heating lamp throughout the experiments. BSP solution (0.2 ml) was injected into the femoral vein over a 15-s period, and bile was collected for 90 min at intervals of 30 min unless otherwise stated. The intravenous dosage of BSP for HPLC studies was 4 mg and for TLC studies 10 mg of the pigment per

body. Bile samples were stored at −80°C until analysis. For the HPLC analysis, the bile samples were appropriately diluted (between 1:1 and 1:50) with distilled water. The resulting solution was directly injected into the HPLC column.

Reproducibilities

Both within- and between-day reproducibilities were tested for BSP, BSP–mGSH(α) and BSP–mMer(a). Three concentrations of each pigment were included in this study. Ten aliquots of each sample were analysed on the same day, and the resulting coefficients of variation (C.V.) indicated the within-day reproducibilities. Aliquots of the same samples were tested once a day for ten days, and the resulting C.V. indicated the between-day reproducibilities.

Detection limit

The limit of detection was defined as the concentration of BSP and BSP–mercaptides resulting in a signal-to-noise ratio of 5.

TLC studies of BSP metabolites in rat bile

Bile samples (25–30 μl) were applied to the starting line marked on a 20 cm × 20 cm cellulose plate (Merck, Type 5716). As described by Whelan and Plaa [6], ascending chromatograms were prepared with the organic solvent phase of *n*-butanol–acetic acid–water (4:1:5, v/v). The plates were developed to a height of *ca.* 17 cm from the starting line. For the detection of BSP metabolites the plate was exposed to concentrated ammonia fumes. The coloured areas of cellulose were scraped off the plate with a flat spatula. The BSP pigments were eluted by shaking the cellulose powder with exactly 0.2 ml of solvent A. The resulting supernatant was used for HPLC analysis of BSP metabolites after appropriate dilution with the same solvent.

RESULTS AND DISCUSSION

Separation of BSP and its mercaptide conjugates

Fig. 1 shows chromatograms obtained from drug-free bile and an artificial bile sample spiked BSP and authentic BSP–mercaptide conjugates.

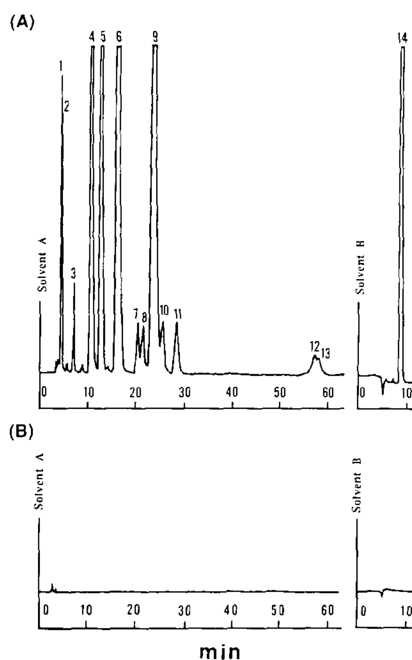

Fig. 1. Representative chromatogram of a standard mixture of authentic BSP–mercaptide conjugates, which were prepared as described in the text. Peaks: 1 = BSP–dGlu-Cys (6.8 pmol) and BSP–dGSH (12.6 pmol); 2 = BSP–dCys (18.4 pmol); 3 = BSP–dCys-Gly (9.2 pmol) and BSP–dMer (2.1 pmol); 4 = BSP–mGlu-Cys(α) (225.3 pmol); 5 = BSP–mGSH(α) (239.0 pmol); 6 = BSP–mCys(α) (292.9 pmol); 7 = BSP–mGlu-Cys(β) (11.8 pmol); 8 = BSP–mMer(α) (10.7 pmol); 9 = BSP–mCys-Gly(α) (252.7 pmol); 10 = BSP–mGSH(β) (17.1 pmol); 11 = BSP–mCys(β) (18.9 pmol); 12 = BSP–mCys-Gly(β) (6.2 pmol); 13 = BSP–mMer(β) (5.0 pmol); 14 = BSP (839.6 pmol). HPLC conditions: Capcell Pak C_{18} column; mobile phase A, 0.1 *M* TEA–H_3PO_4 buffer (pH 9.9 at 22°C)–acetonitrile (90:13, v/v); mobile phase B, 0.1 *M* TEA–H_3PO_4 buffer (pH 9.9 at 22°C)–acetonitrile (73:27, v/v); flow-rate, 1.0 ml/min; detector set at 580 nm; detector sensitivity, 0.01 a.u.f.s.; chart speed, 0.2 mm/min; temperature, 22–23°

Solvents A and B were selected after a series of preliminary experiments. The solvent ratio in system A was chosen to obtain mainly the best resolution of BSP–mercaptide conjugates, and system B was adopted as the best for rapid and good separation of unconjugated BSP from its many metabolites. When 10 μl of undiluted blank bile were directly injected into the liquid chromatograph under the stated conditions, no peaks interfered with the resolution of BSP and its authentic mercaptide conjugates. The authentic

samples of BSP–mercaptide conjugates used in this experiment were the reaction mixtures containing equimolar amounts (0.1 mmol) of BSP and an appropriate mercaptide such as GSH and Cys, and an excess molar amount (4.7 mmol) of 28% ammonium hydroxide in a final volume of 2 ml. The reaction was conducted for 2 h at 37°C, then the mixture was diluted (between 1:1000 and 1:10000) with blank bile and directly injected into the column. In every case, there were three main peaks corresponding to BSP–dimercaptide and two positional isomers of BSP–monomercaptide (data not shown).

In this paper, the positional isomers of BSP–monomercaptide are provisionally distinguished by adding the symbol α or β to the end of each abbreviation, such as BSP–mGSH(α) and BSP–mGSH(β) for the respective isomers of BSP–mGSH, because it remains to be established which of the bromine atoms in the BSP molecule is preferentially displaced by a mercaptide molecule, such as GSH. The formation rate of every α isomer was faster than that of the corresponding β isomer. Moreover, as far as BSP–mGSH is concerned, the biological formation of the α isomer in the rat liver cytosol was faster than that of the β isomer (data not shown). The amounts of the authentic BSP conjugates injected into the HPLC column were calculated from the calibration line for BSP–mGSH (α), which was derived from plots of the peak areas against various concentrations of pure pigment.

Fig. 1 also shows that all the authentic BSP–mercaptide conjugates, except BSP–mMer(β) and BSP–mCys-Gly(β), eluted within 30 min. With solvent A, however, BSP–dGlu-Cys, BSP–dGSH and BSP–dCys coeluted *ca.* 4 min after injection. The retention time for both BSP–dMer and BSP–dCys-Gly was *ca.* 7 min. Moreover, BSP–mMer(β) and BSP–mCys-Gly(β) also coeluted at *ca.* 57 min under the stated conditions. These problems, however, can be solved by means of other solvent systems, as described below.

Fig. 2 depicts typical HPLC patterns of the bile samples from a rat given a single injection of BSP (4 mg). The samples were collected for three con-

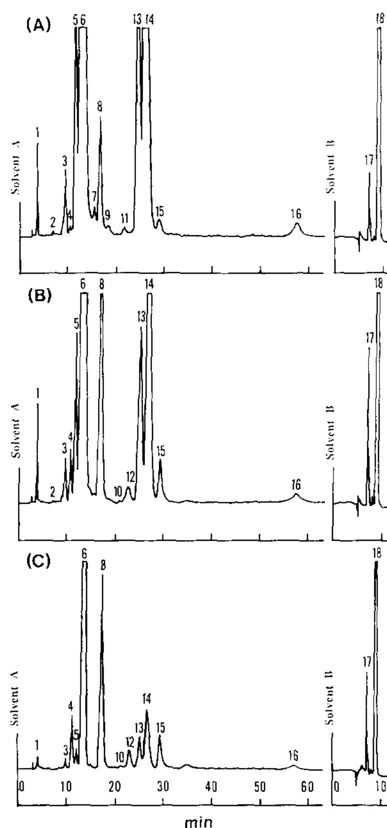

Fig. 2. Typical chromatograms of (A) rat bile collected for 30 min after a single intravenous injection of BSP (4 mg), (B) the same rat bile collected during 30–60 min after BSP injection and (C) the same rat bile collected during 60–90 min after BSP injection. Peaks: 1 = a mixture of BSP–dGSH, BSP–dCys and X (see Fig. 3); 2 = unknown; 3 = unknown; 4 = BSP–mGlu-Cys (α); 5 = unknown; 6 = BSP–mGSH(α); 7 = unknown; 8 = BSP–mCys(α); 9 = unknown; 10 = BSP–mGlu-Cys(β); 11 = unknown; 12 = BSP–mMer(α); 13 = BSP–mCys-Gly(α); 14 = BSP–mGSH(β); 15 = BSP–mCys(β); 16 = BSP–mCys-Gly(β), BSP–mMer(β) and X-2 (see Fig. 4); 17 = unknown; 18 = BSP. HPLC conditions as in Fig. 1.

secutive 30-min periods following the injection. At least eighteen peaks of BSP metabolites were observed in the samples collected in any experimental period. None of the peaks was present in the blank bile. With the addition of known amounts of the authentic BSP–mercaptide conjugates to the bile samples, the elution profile of the chromatogram remained the same except that peaks 1, 4, 6, 8, 10, 12, 13, 14, 15, 16 and 18 each increased by the amount added (data not shown). From these experiments, we assigned the peaks

as follows: peaks 1, 4, 6, 8, 10, 12, 13, 14, 15, 16 and 18 corresponded to a mixture of BSP–dGSH and BSP–dCys, BSP–mGlu-Cys(α), BSP–mGSH (α), BSP–mCys(α), BSP–mGlu-Cys(β), BSP–mMer(α), BSP–mCys-Gly(α), BSP–mGSH(β), BSP-mCys(β), a mixture of BSP–mMer(β) and BSP–mCys-Gly(β), and BSP, respectively. The total amount of the pigments excreted into the bile in 90 min was 84.3% of the dose. About 57% of the dose was BSP–mercaptide conjugates. The main biliary metabolites were the two positional isomers of BSP–mGSH: 48.5% and 3.5% of the dose for peak 6, BSP–mGSH(α), and peak 14, BSP–mGSH(β), respectively. The amount excreted in bile was *ca.* 0.04, 0.2, 0.9, 0.2, 2.3, 0.1, 0.1 and 27.3% of the dose for peaks 1, 4, 8, 12, 13, 15, 16 and 18, respectively. The total amount of unknown metabolites (peaks 2, 3, 5, 7, 9, 11 and 17) excreted in bile was *ca.* 1.2% of the dose.

As shown in Fig. 1, the peaks of some possible BSP–dimercaptide conjugates, and the peaks of BSP–mMer(β) and BSP–mCys-Gly(β), coeluted with solvent A. These problems can be solved with some slight alterations to the solvent system and the flow-rate. Fig. 3A shows the HPLC pattern of five authentic BSP–dimercaptide conjugates, BSP-dGlu-Cys, BSP–dGSH, BSP–dCys, BSP–dMer and BSP–dCys-Gly. The solvent was of 0.1 *M* TEA–H_3PO_4 buffer (pH 9.4)–acetonitrile (85:13, v/v) and the eluent flow-rate was 0.6 ml/min. Fig. 3B and C show the HPLC patterns of bile samples collected for two consecutive 30-min periods following the injection of 4 mg of BSP. These results show that no peaks corresponding to BSP–dGlu-Cys, BSP–dMer and BSP–dCys-Gly were found in the bile samples. The amount excreted in bile during the experimental period was *ca.* 0.05, 0.001 and 0.002% of the dose for BSP–dGSH, BSP–dCys and X, respectively. These results indicate that peak 1 in Fig. 2 mainly arose from BSP–dGSH.

BSP–mCys-Gly(β) and BSP–mMer(β) were also separated more clearly with another mobile phase, namely 0.1 *M* TEA–H_3PO_4 buffer (pH 10.0)–acetonitrile (88:13, v/v) at a flow-rate of 1.0 ml/min (Fig. 4). Fig. 4B and C show the HPLC patterns of BSP–mCys-Gly(β) and BSP–mMer

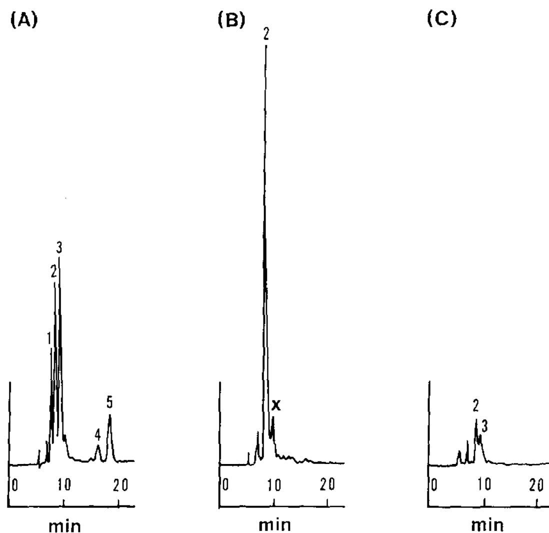

Fig. 3. Chromatograms of (A) a standard mixture containing authentic BSP–dGlu-Cys (7.7 pmol), BSP–dGSH (15.4 pmol), BSP–dCys (22.2 pmol), BSP–dMer (2.1 pmol) and BSP–dCys-Gly (7.6 pmol), (B) rat bile collected for 30 min after a single intravenous injection of BSP (4 mg) and (C) the same rat bile collected during 30–60 min after BSP injection. Peaks: 1 = BSP–dGlu-Cys; 2 = BSP–dGSH; 3 = BSP–dCys; 4 = BSP–dMer; 5 = BSP–dCys-Gly. HPLC conditions as in Fig. 1, except that the mobile phase was 0.1 *M* TEA–H_3PO_4 buffer (pH 9.4 at 22°C)–acetonitrile (85:13, v/v), and the flow-rate 0.6 ml/min.

(β) in the same bile samples collected for the experiments shown in Fig. 3B and C. The amount of BSP–mCys-Gly(β) excreted during the first and the second 30-min periods was 2.8 and 0.5 nmol (0.06 and 0.01% of the dose), respectively. On the other hand, the amount of BSP–mMer(β) excreted in each collection period was 0.3 and 0.6 nmol (0.006 and 0.013% of the dose), respectively.

For the rapid analysis of BSP metabolites, these minor conjugates, BSP–mCys-Gly(β) and BSP–mMer(β), can be disregarded because they constitute less than 0.1% of the total dyes excreted in each collection period after the injection of various amounts (2–10 mg) of BSP (data not shown). If the mobile phase with changed from solvent A to solvent B at *ca.* 35 min after injection of HPLC sample into the column, the minor conjugates coeluted together at the solvent front, and unchanged BSP eluted at *ca.* 9 min. Thus, the total analysis time can be cut from 100

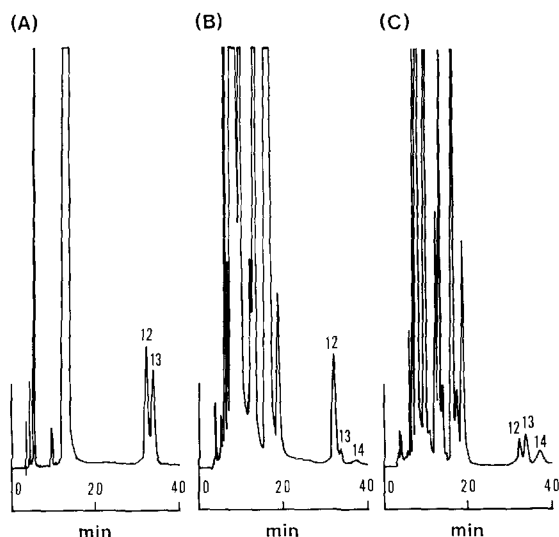

Fig. 4. Chromatograms of (A) a standard mixture containing authentic BSP–mCys-Gly(β) (14.6 pmol) and BSP–mMer(β) (12.0 pmol), (B) rat bile collected for 30 min after a single intravenous injection of BSP (4 mg) in the same experiment as shown in Fig. 3 and (C) the same rat bile collected during 30–60 min after BSP injection. Peaks: 12 = BSP–mCys-Gly(β); 13 = BSP–mMer(β); 14 = unknown (X-2). HPLC conditions were as in Fig. 1, except that the mobile phase was 0.1 *M* TEA–H_3PO_4 buffer (pH 10.0 at 22°C)–acetonitrile (88:13, v/v), and the flow-rate was 1.0 ml/min.

TABLE I

EFFECT OF pH ON HPLC CAPACITY FACTOR

Capacity factor $k' = (t_R - t_0)/t_0$, where t_R = retention time of compound and t_0 = retention time of unretained molecules. HPLC conditions were identical with those in Fig. 1, except that the pH of the buffer in each mobile phase A was adjusted, at 22°C.

BSP–mercaptide conjugate	k'		
	pH 9.80	pH 9.90	pH 10.00
dGlu-Cys	0.53	0.37	0.07
dGSH	0.77	0.40	0.37
dCys	0.97	0.47	0.57
dMer	1.97	1.27	0.90
dCys-Gly	2.12	1.37	0.93
mGlu-Cys(α)	3.53	2.60	1.77
mGSH(α)	4.47	3.27	2.23
mCys(α)	6.13	4.40	3.13
mGlu-Cys(β)	7.27	5.87	4.43
mMer(α)	8.70	6.17	4.33
mCys-Gly(α)	9.77	7.00	4.87
mGSH(β)	10.00	7.63	5.73
mCys(β)	10.43	8.57	6.67
mCys-Gly(β)	22.93	17.93	13.40
mMer(β)	23.33	18.30	13.93

min to *ca.* 60 min, including a 10-min re-equilibration delay.

HPLC conditions

Table I shows that variations in the pH of the TEA–H_3PO_4 buffer in solvent A significantly affected the separation and the retention times of authentic BSP-mercaptide conjugates. Since the pH of buffer is higly sensitive to its temperature, special care should be taken in adjustment of its pH. When the change in pH was measured at various temperatures and expressed as a function of temperature, dpH/d*t* was *ca.* −0.030 unit/degree.

Reproducibilities, linearity and limit of detection

Within- and between-day reproducibilities for BSP, BSP–mGSH(α) and BSP–mMer(α) solutions are shown in Table II. The coefficient of variation (C.V.) of peak areas for each pigment was less than 2%.

The linearity study was carried out with concentrations ranging from 0.2 to 235.8 nmol/ml BSP and its conjugates in bile. Assuming BSP and all of its mercaptide conjugates to have equal molar absorptivities at 580 nm [3], and an equal

TABLE II

PRECISION OF THE CHROMATOGRAPHIC ASSAY

Concentration (ng/ml)	C.V. of peak area (n = 4) (%)		
	BSP–mGSH(α)	BSP–mMer(α)	BSP
Within-day			
50	1.4	1.4	1.6
500	0.3	0.5	0.6
2000	0.1	0.1	0.2
Between-day			
50	1.5	1.5	2.0
500	1.2	1.5	1.8
2000	1.6	2.0	2.0

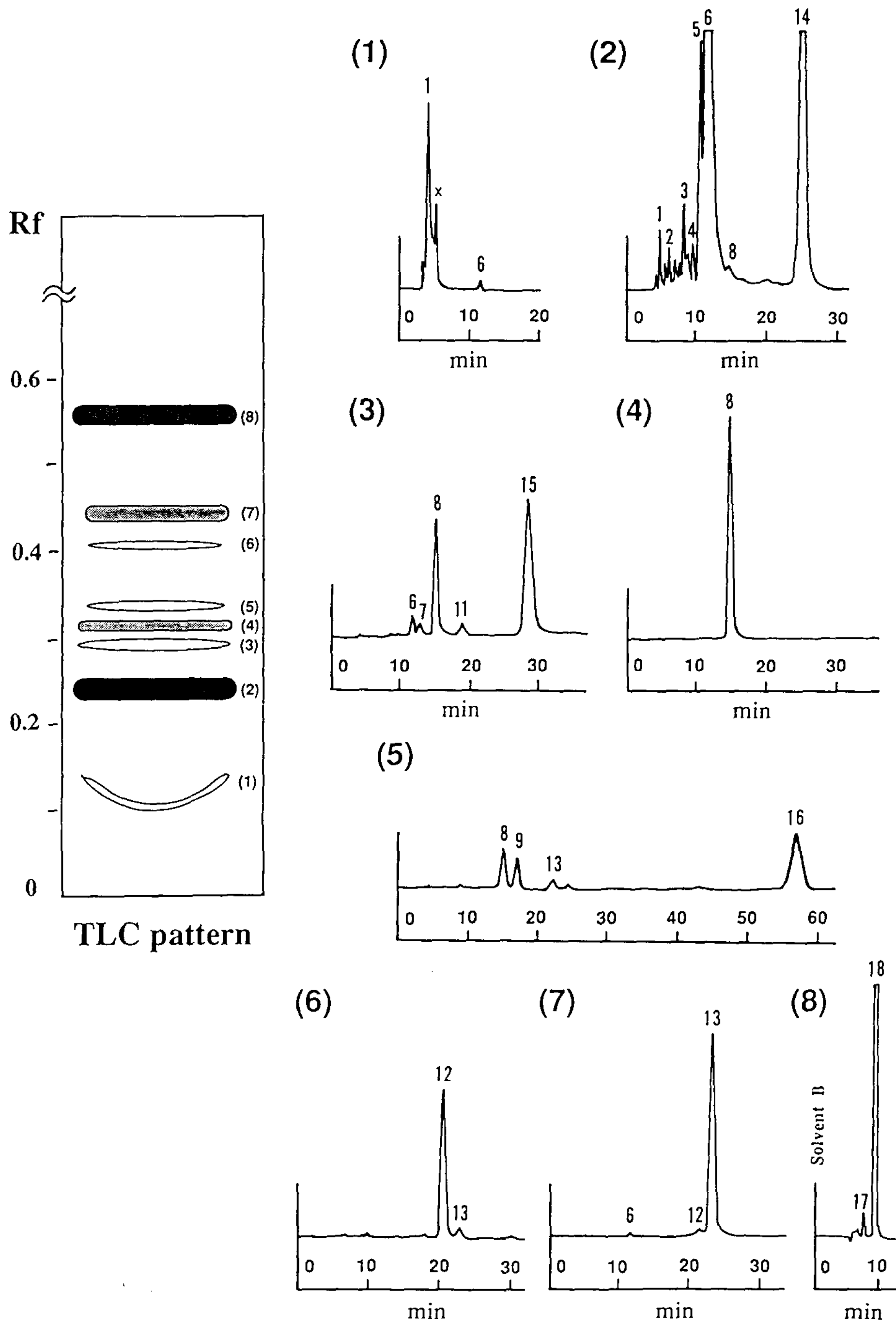

Fig. 5. TLC analyis of BSP metabolites in rat bile and analysis of TLC spots by the HPLC method. TLC spots are identified by the numbers in parenthesis. The peak numbers on the HPLC chromatograms of the TLC spots correspond with those in Fig. 2.

detector response, the amount of each pigment can be determined by only two calibration lines: one for BSP–mercaptide conjugates and another for unchanged BSP. The linearity of the calibration lines obtained by plotting the peak areas against the concentrations (equivalent to ng/ml

BSP) was checked. The calibration lines for BSP–mercaptide conjugates were obtained with authentic BSP–dGSH, BSP–mGSH(α) and BSP–mMer(α) as the representative standards. The equations resulting from least-squares analysis for best fit were $y = 40.05x - 750$ ($r = 0.9998$), $y = 40.08x - 816$ ($r = 0.9999$) and $y = 40.13x - 856$ ($r = 0.9998$), respectively. Similar linearity was obtained with BSP ($r = 0.9992$, $y = 21.5x + 103$).

The limits of detection for BSP–mercaptide conjugates and BSP were *ca.* 0.6 and 2.0 pmol per 10-μl injection, respectively.

In this HPLC method we used an external standard for quantitation of BSP metabolites, since only pipetting error could be expected through the analytical procedure for their assays in body fluids, including bile and plasma. In fact, we can also assay BSP and its metabolites in plasma samples from rats, guinea-pigs and rabbits, In these cases, the plasma samples should be diluted 1:1 with solvent A and centrifuged before assay. The pre-column also should be replaced with a new one after *ca.* 100 injections, to keep the efficiency of the analytical column. The recoveries for the externally added BSP and its metabolites were *ca.* 100% (to be reported elsewhere). It will be possible to use BSP–dMer as an internal standard for the extraction of BSP and its metabolites from some tissues and organs, since the pigment may not be appreciably synthesized in animals (Fig. 3).

Evaluation of the TLC method

The TLC method reported by Whelan and Plaa [6] is acknowledged as the best for the analysis of BSP metabolites. In order to test the homogeneity of the metabolites separated by the TLC method, bile samples collected from a rat dosed with BSP (10 mg) were separated under the same conditions as for the previous method, except for the use of ready-made and coated cellulose glass plates [6]. Fig. 5 shows a typical TLC pattern of the first 30-min bile sample, and the chromatograms obtained from HPLC analysis of each TLC spot. The major BSP metabolites, BSP–mGSH(α), BSP–mGSH(β), BSP–mCys(α), BSP–mCys-Gly(α) and BSP accounted for *ca.* 71, 5, 4, 2 and 14% of the total pigments, respectively. The HPLC analysis proved that every TLC spot contained several pigments other than the main ingredient: BSP–mGSH(α) was found in spots 3, 6 and 7 as well as spot 2, and BSP–mCys(α) was also detected in spots 3, 4, 5 and 6. These results suggest that BSP–mercaptide conjugates probably interact with each other and/or other component(s) of the bile and co-migrate under such TLC conditions.

We found that rats given an intravenous injection of BSP excreted at least twenty BSP–mercaptide conjugates into the bile, several of which have not previously been reported. The HPLC method described may be a useful tool for investigating the biotransformation of BSP, including its position in the mercapturic acid pathway.

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