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Metabolism of sulphobromophthalein I: positional isomers of sulphobromophthalein monoglutathione conjugate

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Abstract

Three positional isomers of sulphobromophthalein glutathione monoconjugate (BSP-mGSH) were detected using a paired-ion HPLC method that employs triethylamine phosphate (TEA-H_3PO_4) as a pairing agent. To confirm that these compounds were glutathione (GSH) conjugates, sulphobromophthalein (BSP) was incubated with a four-fold volume of GSH under alkaline ammonium hydroxide. At least 6 metabolites (3 di-GSH conjugates and 3 isomers of mono-GSH conjugates) were produced under these conditions. The three mono-GSH conjugates were each purified and identified as compounds with a molecular weight of 1020 according to FAB mass spectrometry results. Positional isomers of BSP-GSH were provisionally distinguished via the addition of the symbols α, β and δ to the end of each abbreviation, to reflect the amount of isomers present. Thus, the isomer present in the largest quantity was termed BSP-mGSH(α), the second most abundant isomer was termed BSP-mGSH(β) and the third was termed BSP-mGSH(δ). Interestingly, a species difference was recognized in that rat cytosol GSH S-transferase (GST) primarily produced BSP-mGSH(α), whereas guinea-pig cytosol generated BSP-mGSH(δ), BSP-mGSH(α) and BSP-mGSH(β) equally and rabbit cytosol mainly produced BSP-mGSH(β).

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Introduction

Sulphobromophthalein sodium (BSP) has been used in the clinical assessment of hepatic dysfunction and is a useful tool for the investigation of the hepatobiliary transport mechanisms of drugs (Combes & Schenker 1975; Schwarz et al 1980; Ballatori & Clarkson 1985). In addition, the role of glutathione (GSH) conjugation is important for both detoxification and toxication. GSH conjugation of BSP has been reported in numerous studies, and sulphobromophthalein glutathione monoconjugate (BSP-mGSH) has been used as a model compound to study the dependence of GSH conjugation on hepatic GSH content (Snel et al 1995).

The aim of this study is to clarify the details of BSP metabolism in experimental animal models to allow for safer and more efficient clinical application in man. In a previous study, we developed a novel HPLC method for the determination of BSP-mercaptide conjugates. Separation of the pigments with this method is based on paired-ion chromatography using triethylamine phosphate (TEA-H_3PO_4) as a pairing agent (Sano et al 1992). In this study, this TEA-pairing HPLC method allowed us to identify three positional isomers (α-, β- and δ-) of BSP-mGSH. To identify these unknown metabolites, we sequentially quantitated the amount of chemical conjugates produced when BSP was incubated with glutathione under

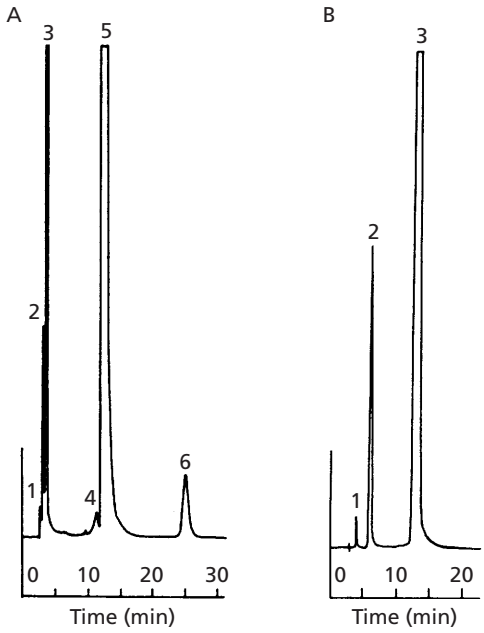

Figure 1 Chromatograms of the HPLC pattern of synthetic BSP-GSH conjugates after incubation for 2 h. Peak 1, BSP-dGSH(β-δ); 2, BSP-dGSH(α-δ); 3, BSP-dGSH(α-β); 4, BSP-mGSH(δ); 5, BSP-mGSH(α); 6, BSP-mGSH(β). HPLC conditions: Capcell Pak C18 column; mobile phases: A, 0.1 M TEA-H_3PO_4 buffer (pH 9.9 at 22°C)–acetonitrile (90:13); B, 0.1M TEA-H_3PO_4 buffer (pH 9.5 at 22°C)–acetonitrile (90:10, v/v) containing 0.1 mM tetra-n-butylammonium bromide; flow rate, 1.0 mL min^{-1}; detector set at 580 nm; detector sensitivity, 0.01 aufs; chart speed, 0.2 mm min^{-1}; temperature, 22–23°C.

alkaline ammonium, and determined the chemical characteristics of these BSP-mGSH isomers.

Materials and Methods

Chemicals

BSP was obtained from Aldrich Chemical Co. (Milwaukee, WI). Reduced GSH and γ-glutamylcysteine (Glu-Cys) were purchased from Kozin Chemical Co. (Tokyo, Japan). Cysteinylglycine (Cys-Gly) was obtained from Bachem Feinchem, AG (Bubendorf, Switzerland). Cysteine (Cys) and *N*-acetylcysteine (Mer) were obtained from Kanto Chemical Co. (Tokyo, Japan). All other chemicals were of reagent grade without further purification.

Standard BSP-mercaptides

Authentic BSP monomercaptides including α-, β- and δ-positional isomers (BSP-mGSH, -dGSH, -mGlu-Cys, -mCys-Gly, -mCys and -mMer) were all synthesized using a modification of the method of Whelan et al (1970) and purified as described in a previous paper (Sano et al 1992).

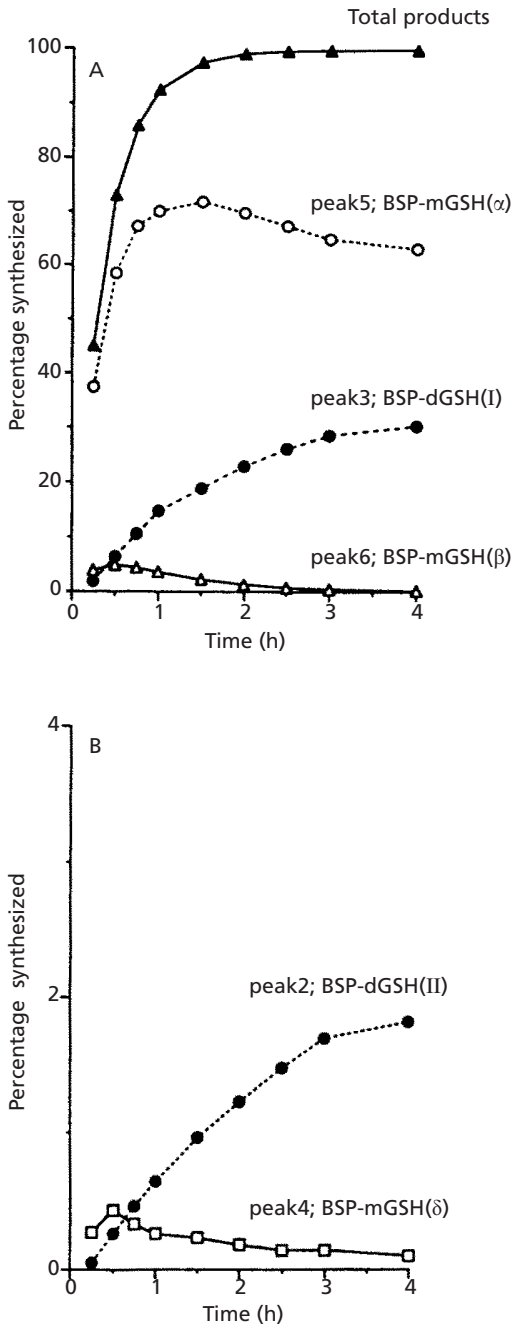

Figure 2 Time-course for synthesis of BSP-GSH conjugates in-vitro. Results are expressed as a percentage of the total amount of BSP-GSH conjugates and unconjugated BSP in the incubation mixture.

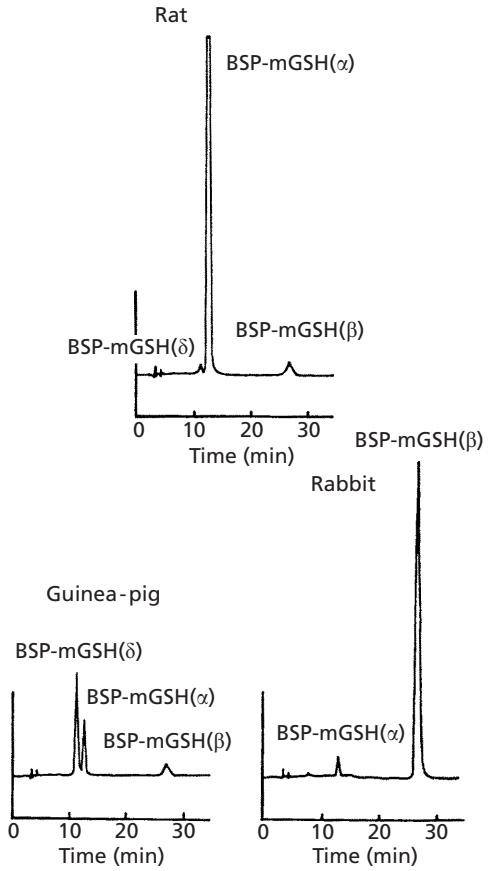

Figure 3 HPLC patterns of BSP-mGSH isomers synthesized by rat, guinea-pig and rabbit cytosol GST. The reaction mixtures contained 0.1 mM of BSP, 10 mM of GSH and 0.5 mg cytosolic protein in 1.0 mL of 0.1 M Tris-HCl (pH 7.5).

Preparation of rat, guinea-pig and rabbit liver cytosol

Male Wistar rats, 230–250 g (Sankyo Labo Service Co. Ltd, Tokyo, Japan), Hartley guinea-pigs, 370–390 g (Sankyo Labo, Japan), and Japanese white rabbits, 1.5 kg (Sankyo Labo, Japan), were housed in stainless steel cages in groups of four or five, under a 12-h light–dark cycle in a temperature controlled room. The animals were allowed free access to standard chow (Sankyo Labo, Japan) and water before the experiments.

Rat, guinea-pig and rabbit livers were quickly excised following perfusion with saline and homogenized with 3 volumes of 1.15% KCl. The homogenate was centrifuged at 105000 *g* for 60 min at 4°C. The supernatant was recovered and stored at −80°C. Protein concentrations were determined according to the method of Lowry et al (1951) with bovine serum albumin used as the standard.

Assay of GST activity

GST activity toward BSP was measured according to the method of Habig et al (1974). The reaction mixtures contained 0.1 mM of BSP and 10 mM of GSH in a final volume of 1.0 mL, at pH 7.5. The supernatant fraction of liver (from 105000 *g* spin) was the source of the enzyme. After incubation (37°C for 5 min), a sample was added to an equivalent volume of ice-cold 20 mM *N*-ethylmaleimide containing 13% (v/v) of acetonitrile, and the mixture was centrifuged at 15000 *g* for 5 min. The supernatant was used as a sample for HPLC.

Hydrolysis by γ-glutamyltranspeptidase (γ-GT) and dipeptidases

Hydrolysis was conducted by partially purified γ-glutamyltranspeptidase (γ-GT) and dipeptidases from

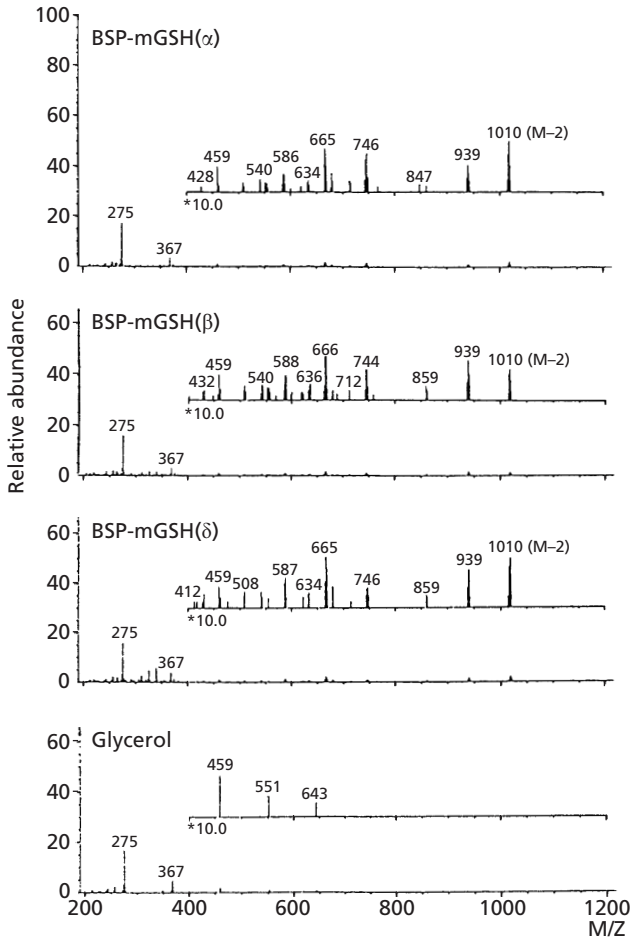

Figure 4 FAB mass spectra of three positional isomers of BSP-mGSH. The spectra were corrected for the glycerol matrix. Some fragments were assigned tentatively.

each animal according to the methods of Hinchman & Ballatori 1990. γ-GT hydrolysis was conducted by incubation with each enzyme fraction at 25°C in the presence of triethanolamine-HCl (0.1 M, pH 9.0), corresponding BSP-mGSH as a substrate, glycylglycine (50 mM) and $MgCl_2$ (10 mM). Dipeptidase hydrolysis was held by incubation with each enzyme fraction at 37°C in the presence of tris-HCl (0.1 M, pH 7.4) and corresponding BSP mCys-Gly as a substrate.

HPLC Assay of BSP and its conjugates

BSP and its conjugates were analysed according to a previous method (Sano et al 1992). HPLC conditions are shown in Figure 1.

FAB mass spectrometry

FAB mass spectrometry was performed on a Joel JMS-AX500 mass spectrometer. The matrix used was glycerol and the target was bombarded with 3 kV Ar gas.

NMR spectroscopy

For NMR measurements, samples were dissolved in D_2O. ^1H and ^{13}C NMR spectra were recorded on a JEOL JMN-LA 500 spectrometer, operating at 500 MHz for ^1H and 125 MHz for ^{13}C.

Results and Discussion

A total of 25 μmol of BSP as a substrate was incubated with a four-fold volume of GSH (100 μmol) in 1 mL of a reaction mixture containing 7.34 mmol of ammonium hydroxide at 37°C. The reaction was stopped via the addition of 20 μL of reaction mixture to 200 μL of 20 mM *N*-ethylmaleimide and 13% (v/v) of acetonitrile. Following centrifugation at about 10000 *g* for 10 min, samples (5–20 μL) of the supernatant were applied onto the HPLC column (Figure 1A). Figure 1B shows detailed chromatograms of di-GSH conjugates using tetra-n-butylammonium bromide in the HPLC method. At least 6 metabolites (3 di-GSH conjugates and 3 mono-GSH conjugates) were produced under these conditions. To date, although mono-GSH isomers have been detected as only one spot on conventional paper chro-

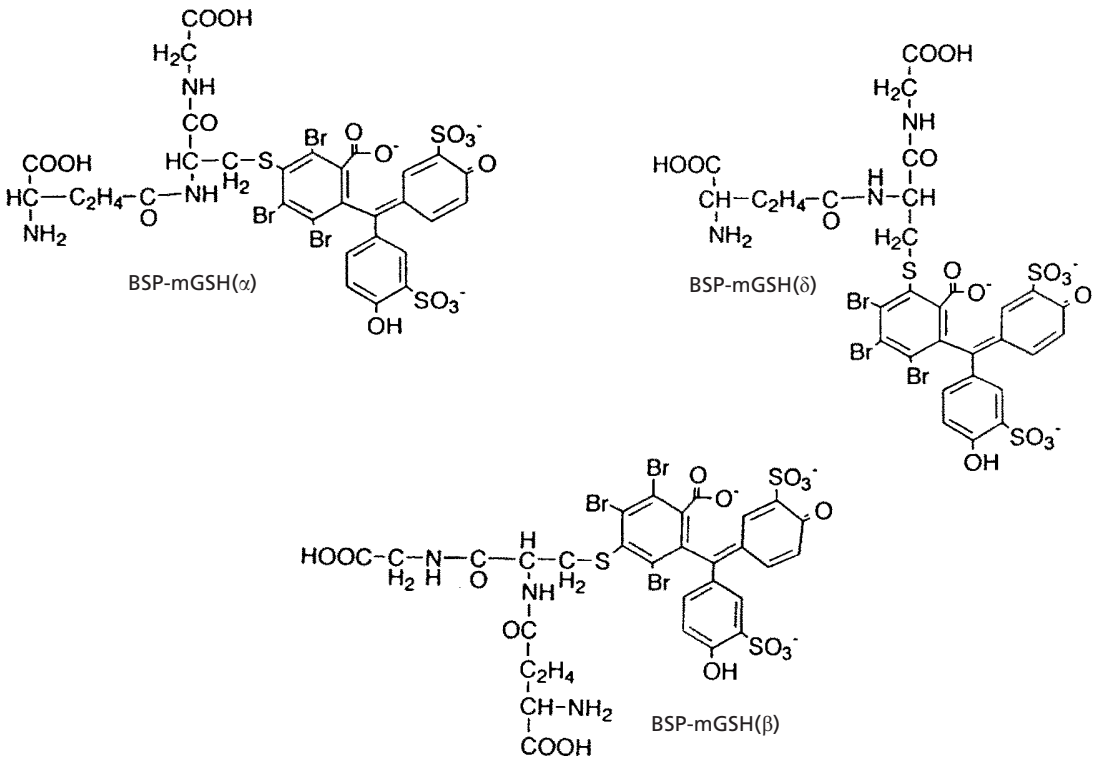

Figure 5 Putative structures of three positional isomers of BSP-mGSH.

matography and TLC (Grodsky et al 1959; Combes & Stakelum 1961; Whelan & Plaa 1963), our HPLC method has identified three isomers located at the same position (Sano et al 1992).

Positional isomers of BSP-GSH were provisionally distinguished via the addition of the symbols α, β and δ to the end of each abbreviation, to reflect the amount of isomer present. Thus, the isomer present in the largest amount (peak 5) was termed BSP-mGSH(α), the second most abundant isomer (peak 6) was termed BSP-mGSH(β) and the third (peak 4) was termed BSP-mGSH(δ). We carried out the following experiments to verify that all three isomers were mono-GSH conjugates. The reason why δ was used for the third isomer instead of γ was to avoid any confusion with γ-GT.

Formation of BSP-mercaptide isomers by chemical reactions

The biosynthesis rates of the α-, β- and δ-isomers of mGSH and dGSH isomers are presented in Figure 2.

As shown in Figure 2A, the amount of α-isomer increased once the reaction had begun and reached C_{max} at 1.5 h. After that point, it decreased gradually. In the same manner, the β-isomer increased to C_{max} in 30 min and then began to decrease. During the same period, the dGSH isomer (I) simultaneously increased.

These results suggest that the dGSH isomer (I) comes from mGSH of the α- and β-isomers. In a similar manner, biosynthesis of the second dGSH isomer (II) was accompanied by a decrease in the δ-isomer (Figure 2B), which also suggests that the δ-isomer was one of the mGSH isomers. Moreover, a new di-GSH conjugate (peak 1) was detected by the method using tetra-n-butylammonium bromide (Figure 1B). This was considered to be a di-GSH conjugate of mGSH(β) and (δ).

Evidence of positional isomer synthesis of BSP-mercaptides by enzymatic reaction

To confirm that peaks 4–6 represented GSH conjugates, we attempted to identify these peaks using GSH conjugates of BSP in the cytosol of rats, guinea-pigs and rabbits. Figure 3 shows the HPLC separation results after 5 min of reaction using each animal's cytosol.

Rat cytosol GST primarily produced BSP-mGSH(α) (2.01 nmol min^{-1} mg^{-1}). Interestingly, guinea-pig cytosol synthesized all BSP-mGSH(α), BSP-mGSH(β) and BSP-mGSH(δ) (0.18, 0.09 and 0.31 nmol min^{-1} mg^{-1}, respectively), whereas rabbit cytosol mainly produced BSP-mGSH(β) (1.13 nmol min^{-1} mg^{-1}). Hydrolysis by γ-GT and dipeptidases demonstrated that all of the corresponding Cys-Gly- and Cys-conjugates were produced. These results indicated that peaks 4–6 were real glutathione conjugates.

Chemical characteristics of BSP-mGSH(α),(β) and (δ) isomers

BSP-mGSH (α), (β) and (δ) were purified using the ODS column. The three compounds were identified as mono-GSH conjugates with a molecular weight of 1020 according to the FAB mass spectrometry results (Figure 4).

The proton NMR data of the glutathionyl moiety were similar to the data of Koppele et al (1986) (2.05(2H, m, Glu-β-CH_2), 2.27(2H, m, Glu-γ-CH_2), 3.05(1H, Cys-CH), 3.26(1H, Cys-CH), 3.69(1H, t, Glu-CH), 3.84(2H, s, Gly-CH_2), 4.27(1H, d of d, Cys-CH)). However, we

Table 1 HPLC capacity factor (k′).

Peak No.	BSP-mercaptide conjugates	k′ (pH 9.90)
1	dGSH	0.40
2	dCys	0.47
3	dCys-Gly	1.37
4	mGlu-Cys(δ)	1.90
5	mGlu-GSH(α)	2.20
6	mGlu-Cys(α)	2.60
7	mGSH(δ)	2.73
8	mGSH(α)	3.27
9	mCys(δ)	4.12
10	mCys(α)	4.40
11	mMer(δ)	5.01
12	mGlu-Cys(α)	5.87
13	mCys-Gly(δ)	6.10
14	mMer(α)	6.17
15	mCys-Gly(α)	7.00
16	mGSH(β)	7.63
17	mCys(β)	8.57
18	mCys-Gly(β)	17.93
19	mMer(β)	18.30

Capacity factor, k' = (tR − t0), where tR is the retention time of the compound and t0 is the retention time for unretained molecules. HPLC conditions: Capcell Pak C18 column; mobile phases: A, 0.1 M TEA-H_3PO_4 buffer (pH 9.9 at 22°C)–acetonitrile (90:13); B, 0.1M TEA-H_3PO_4 buffer (pH 9.5 at 22°C)–acetonitrile (90:10, v/v) containing 0.1 mM tetra-n-butylammonium bromide; flow rate, 1.0 mL min^{-1}; detector set at 580 nm; detector sensitivity, 0.01 aufs; chart speed, 0.2 mm min^{-1}; temperature, 22–23°C.

could not definitively determine which bromine molecule was replaced by glutathione from ^{13}C NMR data (data not shown).

To estimate the binding site, the most possible conformation of each conjugate was obtained by molecular mechanics 2 (MM2) and the SPARTAN molecular orbital calculation method (Figure 5).

Capacity factors (k′) of authentic standard samples

BSP was incubated with four other mercaptides, including cysteine (Cys), cysteinylglycine (Cys-Gly), glutamylcysteine (Glu-Cys) and *N*-acetylcysteine (Mer), as well as GSH. The δ-form of each conjugate was identified chemically. Table 1 shows the capacity factors (k′) of each mercaptide of BSP. The k′ of the dGSH values of only the main (α-β) form is shown in Table 1, because the amount of the other di-GSH conjugates excreted was negligible. All peak numbers are also given. This table of HPLC capacity factors (k′) will be useful to identify peaks of HPLC chromatograms in follow-up studies.

In summary, these findings demonstrated that all three positional isomers of BSP-mGSH were compounds with a glutathionyl moiety and a molecular weight of 1020. We also showed the most possible conformation for the binding site of each conjugate. Interestingly, we observed a species difference in the metabolism of BSP between the rat, guinea-pig and rabbit. Based on the findings of this study, we will investigate the cause of this species difference in the metabolism of BSP in subsequent studies.

References

Ballatori, N., Clarkson, T. W. (1985) Sulfobromophthalein inhibition of glutathione and methylmercury secretion into bile. *Am. J. Physiol.* **248**: G238–G245

Combes, B., Schenker, S. (1975) Laboratory tests-dye tests. In: Schiff, L. (ed.) *Diseases of the Liver*. J. B. Lippincott Co., Toronto, pp 212–246

Combes, B., Stakelum, G. S. (1961) A liver enzyme that conjugates sulfobromophthalein sodium with glutathione. *J. Clin. Invest.* **40**: 981–988

Grodsky, G. M., Carbone, J. V., Fanska, R. (1959) Identification of metabolites of sulfobromophthalein. *J. Clin. Invest.* **38**: 1981–1988

Habig, W. H., Pabst, M. J., Jakoby, W. B. (1974) Glutathione S-transferases. *J. Biol. Chem.* **249**: 7130–7139

Hinchman, C. A., Ballatori, N. (1990) Glutathione-degrading capacities of liver and kidney in different species. *Biochem. Pharmacol.* **40**: 1131–1135

Koppele, J. M., Mark, E. J., Boerrigter, J. C. O., Brussee, J., Gen, A., Greef, J. Mulder, G. J. (1986) α-Bromoisovalerylurea as model substrate for studies on pharmacokinetics of glutathione conjugation in the rat I. *J. Pharmacol. Exp. Ther.* **239**: 898–904

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275

Sano, K., Kinosita, I., Mihara, R., Ikegami, Y., Uesugi, T. (1992) High-performance liquid chromatographic determination of sulphobromophthalein and its conjugates. *J. Chromatogr.* **578**: 63–70

Schwarz, L. R., Götz, R., Klaassen, C. D. (1980) Uptake of sulfobromophthalein-glutathione conjugate by isolated hepatocytes. *Am. J. Physiol.* **239**: C118–C123

Snel, C. A. W., Pang, K. S., Mulder, G. J. (1995) Glutathione conjugation of bromosulfophthalein in relation to hepatic glutathione content in the rat in vivo and in the perfused rat liver. *Hepatology* **21**: 1387–1394

Whelan, F. J., Plaa, G. L., (1963) The application of thin layer chromatography to sulfobromophthalein metabolism studies. *Toxicol. Appl. Pharmacol.* **5**: 457–463

Whelan, G., Hoch, J., Combes, B. (1970) A direct assessment of the importance of conjugation for biliary transport of sulfobromophthalein sodium, *J. Lab. Clin. Med.* **75**: 542–557