Disposition of the Bromosulfophthalein-Glutathione Conjugate in the Isolated Perfused Rat Kidney

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Abstract

Renal elimination of the bromosulfophthalein-glutathione conjugate (BSP-GSH) after its i.v. administration in the rat in vivo is negligible. In our study we wanted to establish whether the high albumin-binding of BSP-GSH constitutes the major restrictive factor toward the urinary excretion of the compound. The renal disposition of BSP-GSH was studied in the isolated rat kidney during perfusions with or without albumin in the perfusate. The urinary clearance of BSP-GSH in the absence of albumin was very low (<60 μl/min) as compared to the inulin clearance (approximately 300 μl/min). This indicates that albumin-binding is not the major reason for the low urinary clearance of BSP-GSH. Addition of albumin to the perfusate further decreased the urinary excretion by 60%. BSP-GSH is metabolized by the kidney into two major metabolites: the cysteinylglycine conjugate and the di-glutathione conjugate. Both metabolites appear in urine after renal biotransformation to mercapturic acid. The di-glutathione conjugate is further metabolized to the di-cysteinylglycine conjugate. The di-glutathione conjugate and the di-cysteinylglycine conjugate are the major urinary components and the urinary elimination of BSP-GSH may depend on their formation. Inhibition of γ-glutamyl transpeptidase activity with acivicin largely prevented the degradation to the cysteinylglycine and dicysteinylglycine conjugates of BSP. The total rate of urinary excretion, however, was only slightly lowered by acivicin. Apparently, cleavage of the γ-glutamyl moiety is not relevant for the total urinary elimination of BSP-GSH.

The liver diagnostic dye BSP is commonly used as model compound for studies on hepatic uptake mechanisms and as substrate for GSH conjugation. In the rat, BSP is metabolized in the liver to yield the BSP-GSH conjugate (Krebbs and Brauer, 1958; Grodsky et al., 1959; Combes and Stakelum, 1960; Combes, 1965; Whelan et al., 1970). Degradation of BSP-GSH to cysteinylglycine and cysteine conjugates, as well as the formation of di-GSH conjugates and derived metabolites, has been reported (Sano et al., 1992), but these pathways are quantitatively negligible. BSP and BSP-GSH are both tightly bound to albumin (Baker and Bradley, 1966; Pfaff et al., 1975), and their hepatic uptake is thought to proceed via an albumin-mediated uptake mechanism (Levi et al., 1969; Scharschmidt et al., 1975; Weisiger et al., 1981; Theilmann et al., 1984).

In the rat in vivo, BSP is mostly eliminated by the liver (Klaassen, 1975; Yam et al., 1976; Snell et al., 1995). The same observation has been made for the BSP-GSH conjugate: when BSP-GSH is administered i.v., the dose is virtually completely recovered in bile, mostly as unchanged compound (Yam et al., 1976; Snell et al., 1995). Urinary elimination of BSP-GSH appears to be a very minor excretory pathway. In this respect, the disposition of BSP-GSH differs from that of most other GSH conjugates, which are readily excreted in urine after renal biotransformation to mercapturic acid (Inoue et al., 1992). Unlike many other GSH conjugates, BSP-GSH is tightly bound to albumin: 99% is bound under physiological conditions (Baker and Bradley, 1966). When the dissociation rate of BSP-GSH from albumin is low in comparison to the renal transit rate of albumin-bound BSP-GSH through the glomeruli, albumin-binding may be the reason why BSP-GSH is poorly filtered by the glomeruli and why the urinary excretion is very small.

In our study, we wanted to establish whether albumin-binding is indeed the reason for the poor renal elimination of BSP-GSH. The disposition of BSP-GSH was studied in the isolated perfused rat kidney during perfusions with or without albumin in the perfusate.

Methods

Chemicals. BSP was purchased from Janssen Chimica, Geel, Belgium. Reduced GSH was from E. Merck, Darmstadt, Germany. Acivicin [L-(αS,βS)-α-amino-3-chloro-4,5-dihydro-5-isoxazoletenic acid] was purchased from Sigma Chemicals. Other chemicals were of highest purity from standard sources.

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ABBREVIATIONS: BSP, bromosulfophthalein; GSH, glutathione; BSP-GSH, glutathione conjugate of BSP; BSP-cys-gly, cysteinylglycine conjugate of BSP; BSP-(GSH)₂, diglutathione conjugate of BSP; BSP-(cys-gly)₂, dicysteinylglycine conjugate of BSP; HPLC, high-performance liquid chromatography.
Renal disposition of BSP-GSH

BSP-GSH, a low molecular weight compound, was administered in two groups: one contained 2.5% pluronic F108 (albumin-free) and the other, 2.2% pluronic F108 plus bovine serum albumin (albumin-containing) during (5,000 pores) the experiment. The solution was oxygenated with a bubble flowmeter and a flow-through pH meter, respectively. The GFR was calculated on the basis of inulin clearance as follows: GFR = (inulin in perfusate/ inulin perfusate) x urine flow rate. Inulin concentrations in urine and perfusate were determined by the method of Heyrovsky (1956).

The experimental period lasted for 90 min, during which urine samples were collected at 10-min intervals. Perfusion samples (250 µl) were taken from the reservoir at the T_{mid} of the urine collection interval. An additional perfusate sample was taken at the end of the perfusion. Perfusion and urine samples were stored at −20°C until analysis.

Analytical methods. BSP-GSH in perfusate was quantitated by a previously reported HPLC method (Snel et al., 1992), but with some minor modifications. The separation was achieved on a stainless steel column (150 x 3 mm i.d.), packed with 5-µm particles of Spherisorb ODS2, reversed phase material. The system comprised a S1000 solvent delivery system, a S2110 low pressure mixer, a S2000 HPLC controller (Sykam, Gauting, Germany) and a Promin II autosampler (Separations Analytical Instruments, Emmen, The Netherlands). Elution solvent A was a 10 mM sodium phosphate buffer, pH 6.0; solvent B was acetonitrile. The following elution profile was used at a constant flow rate of 0.55 ml/min; isocratic conditions (0% B) were maintained for 2.5 min, followed by a linear gradient to 30% B in 15 min. Hereafter the system was reverted to the initial conditions in 2.5 min and allowed an equilibration interval of 5 min for the next injection. UV detection was achieved with a spectrophotometer (Waters Associates Inc., Milford, MA) at a wavelength of 254 nm.

Perfusion samples without albumin were diluted 10-fold with 10 mM sodium phosphate buffer, pH 6.0. The diluted samples were directly injected onto the HPLC system. The albumin-containing perfusate samples were first mixed with a saturated urea solution (100 µl sample + 100 µl urea solution). Hereafter, proteins were precipitated by addition of 800 µl acetonitrile and subsequent centrifugation; 900 µl of the supernatant were transferred to another tube and dried under a stream of nitrogen gas. The residue was reconstituted in 900 µl 10 mM sodium phosphate buffer, pH 6.0. The obtained solution was injected onto the HPLC. A series of BSP-GSH solutions (10–100 µM) in blank perfusate (with or without albumin) was processed as described above for the experimental samples and furnished the calibration curve.

Urine and perfusate samples were also analyzed spectrophotometrically at 578 nm without prior separation of the compounds that absorb at this wavelength. Samples were diluted 10-fold with a 0.1 N sodium hydroxide solution and the absorbance was read at 578 nm. A series of BSP-GSH solutions (10–100 µM) in blank urine or blank perfusate was diluted as described for the experimental samples and furnished the calibration curve. For quantitative purposes, it was assumed that BSP-GSH derived metabolites possess the same molar extinction coefficient as BSP-GSH.

Identification of BSP-GSH-derived metabolites. For the identification of BSP-GSH-derived metabolites in perfusate and urine, a higher degree of selectivity of detection was needed. Therefore, the HPLC separation methodology was combined with post-column alkalization and spectrophotometric detection of the purp­le chromophores at 578 nm, a principle previously described by Van’t Klooster et al. (1988). A 0.05 N sodium hydroxide solution in water/acetonitrile = 80/20 (v/v) was delivered by a HPLC pump (Water Associates Inc., Milford, MA) at a flow rate of 0.2 ml/min, and added to the eluent from the column via a T connection. A mixing coil was placed between the T connection and the detector. Highly selective detection of BSP-GSH and its metabolites was achieved with a variable wavelength Spectroflow 757 spectrophotometer (Kratos Analytical, Ramsey, NJ) at a wavelength of 578 nm.

This method of detection was also used to check the stability of BSP-GSH in perfusate and in urine samples: blank albumin-containing and albumin-free perfusate (perfused through the kidney for 20 min), and blank urine were spiked with BSP-GSH (37 µM) and incubated at 37°C. Samples were taken every hour for a total period of 6 h, and immediately processed as described above for HPLC
analysis. BSP-GSH and BSP-(GSH)$_2$ (which was present as a minor chemical impurity) were found to be stable under these conditions.

**Protein binding of BSP-GSH.** Unbound BSP-GSH in albumin-containing perfusate was isolated by ultrafiltration. Albumin-containing perfusate was spiked with BSP-GSH (37 μM) and incubated for 1 h at 37°C. The perfusate was then centrifuged at 37°C for 30 min at 2000 × g through YMT membranes (Amicon micropartition system; Amicon Inc., Beverly, MA). The BSP-GSH concentration in total perfusate and ultrafiltrate samples was determined spectrophotometrically as described above. The unbound fraction was calculated as: [BSP-GSH] in ultrafiltrate/[BSP-GSH] in total perfusate.

**Data analysis.** The statistical significance of differences between the experimental groups was evaluated, using an analysis of variance test. A comparison for each individual time point was made between the experiments with albumin-free perfusate and albumin-containing perfusate, and between the experiments with albumin-free perfusate and albumin-free/acivicin-containing perfusate. A comparison between the experiments with albumin-containing perfusate and albumin-free/acivicin-containing perfusate was omitted. A P < .05 was considered significant.

The renal clearance of BSP-GSH (Cl$_r$) was calculated in two ways:

\[
\text{Cl}_r = \left( \frac{\text{[BSP - GSH + metabolites]}_{\text{urine}}}{\text{[BSP - GSH]}_{\text{perfusate}}} \right) \times \text{urine flow rate.}
\]

\[
\text{Cl}_r = \left( \frac{\text{[BSP - GSH + metabolites]}_{\text{urine}}}{\text{[BSP - GSH + metabolites]}_{\text{perfusate}}} \right) \times \text{urine flow rate.}
\]

**Results**

**Evaluation of renal function during the kidney perfusions.** Renal function was monitored during the control and experimental period by determination of GFR, urine flow rate and urinary pH (fig. 1). Throughout the control and experimental period, the urinary pH and GFR were not significantly different between the compared groups. During the last 30 min of the experimental period (60–90 min), the urine flow rate was significantly higher in perfusion with albumin-free/acivicin-containing perfusate than in perfusions with albumin-free perfusate without acivicin. The GFR and the urine flow rate did not change in the course of the perfusion. The urinary pH, however, showed a tendency to increase in all perfusions.

**Elimination of BSP-GSH from perfusate: effect of albumin.** Kidney perfusions were conducted with or without albumin in the perfusate. The BSP-GSH concentration in the recirculating perfusate medium decreased rapidly in the absence of albumin (fig. 2a). This was due to its conversion to metabolites: the total concentration of BSP-GSH plus its metabolites in perfusate decreased only very little during the perfusion (fig. 2b). Albumin decreased the rate of disappearance of BSP-GSH from the recirculating perfusate (fig. 2a); from 30 to 90 min, differences between the two experimental groups in the BSP-GSH concentration in perfusate were statistically significant. Albumin, however, did not affect the rate of disappearance of BSP-GSH plus its metabolites (fig. 2b); the concentration-time profiles were virtually identical for the two experimental groups.

To identify the BSP-GSH derived metabolites, the recirculating perfusate at the end of the perfusion was analyzed by HPLC with post-column alkalization and spectrophotometric detection at 578 nm. This procedure revealed the presence of two BSP-GSH-derived metabolites (fig. 3); with the aid of reference compounds, they were identified as the disubstituted BSP-(GSH)$_2$ and the cysteinylglycine conjugate of BSP (BSP-cys-gly). The relative contribution of the components was different for the two experimental groups: the contribution of BSP-cys-gly appeared to be higher in albumin-free than in albumin-containing perfusate.

**Excretion of BSP-GSH and its metabolites in urine: effect of albumin**

Total urinary excretion rates (without differentiation between BSP-GSH and its metabolites) were estimated spectrophotometrically at 578 nm after alkalization of the urine samples. Throughout the experiment, excretion rates were significantly higher in perfusions with albumin-free perfusate than in perfusions with albumin-containing perfusate.
Acivicin decreased the rate of disappearance of BSP-GSH from perfusate (fig. 2a); differences between the two groups were significant from 20 to 90 min of the experimental period. The total concentration of BSP-GSH and its metabolites in perfusate decreased very little during the perfusion (fig. 2b); the concentration-time profile did not differ between the two groups. Application of the HPLC post-column alkalization procedure demonstrated that BSP-cys-gly was absent from the perfusate at the end of the perfusion (fig. 3); perfusate only contained BSP-GSH and BSP-(GSH)₂.

Total urinary excretion rates (without differentiation between the urinary metabolites) were lower, but not significantly different from the excretion rates in perfusates without acivicin (fig. 4). The urinary metabolite pattern, however, differed considerably between the two experimental groups (table 1): in perfusions with acivicin, BSP-GSH and BSP-(GSH)₂ accounted for 75% of the total amount in urine, whereas in absence of acivicin, their contribution amounted to only 15% and the corresponding cysteinylglycine conjugates were predominant.

**Protein binding of BSP-GSH in perfusate.** The unbound fraction of synthetic BSP-GSH in albumin-containing perfusate was found to be 14%.

### Discussion

The urinary elimination of BSP-GSH was clearly restricted by binding to albumin. The urinary BSP-GSH clearance (fig. 5a) in the presence of albumin was approximately 20% of that in the absence of albumin in the perfusate; this seems to agree reasonably well with the 14% unbound fraction. However, albumin-binding cannot account for the low urinary clearance: in the absence of albumin, the total urinary clearance of BSP-GSH was still very low (maximally 60 μl/min; see fig. 5a), compared to that of inulin (260–310 μl/min). Unless for some unknown reason BSP-GSH is not filtered in the glomeruli, this suggests that tubular reuptake of BSP-GSH plays an important role. This seems to be supported by the fact that the urinary clearance of BSP-GSH (mainly in the form of metabolites) slowly increased in the course of the perfusion, whereas that of inulin was more or less constant: a possible explanation for the increase of the urinary clearance is that the metabolites that accumulated in the course of the perfusion were more efficiently excreted in urine than unchanged BSP-GSH.

BSP-cys-gly and BSP-(GSH)₂ appeared to be the major metabolites of BSP-GSH formed in the kidney. BSP-cys-gly is probably generated in the lumen of the proximal tubule, because its formation is catalyzed by γ-glutamyl transpeptidase, an enzyme that is abundantly present in the brush-border membrane at the apical side of proximal tubular cells (Rutenberg et al., 1969; Glossmann and Neville, 1972). However, BSP-cys-gly is not only recovered in urine, but also in perfusate. Therefore, it seems that BSP-cys-gly undergoes tubular reuptake from the lumen or that BSP-cys-gly is formed during reuptake of BSP-GSH. Alternatively, BSP-cys-gly in the perfusate may be formed by γ-glutamyl transpeptidase at the basolateral side of the cells.

The formation of the BSP-(GSH)₂ conjugate must take place intracellularly, possibly in the proximal tubular cells. This requires (tubular re-)uptake of BSP-GSH. BSP-(GSH)₂ appears in the perfusate and probably undergoes glomerular...
filtration and degradation to BSP-(cys-gly)$_2$ in the lumen of the proximal tubule. Although tubular reuptake of BSP-(cys-gly)$_2$ is unlikely, because this metabolite was not detected in the perfusate at the end of the perfusion, uptake by the tubular cells followed by local sequestration or metabolism cannot be ruled out with certainty.

BSP-(GSH)$_2$ and BSP-(cys-gly)$_2$ constitute the major urinary components. The urinary clearance of BSP-GSH may largely depend on their formation.

To study whether $\gamma$-glutamyl transpeptidase catalyzed degradation interferes with the renal clearance of BSP-GSH, kidney perfusions were conducted with the $\gamma$-glutamyl transpeptidase inhibitor acivicin in albumin-free perfusate. Acivicin effectively prevented the conversion of BSP-GSH

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**Table 1**

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<thead>
<tr>
<th>Urinary Metabolite</th>
<th>Perfusion Composition</th>
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<tbody>
<tr>
<td></td>
<td>2.5% pluronic F108</td>
</tr>
<tr>
<td>BSP-(GSH)$_2$</td>
<td>9</td>
</tr>
<tr>
<td>BSP-(cys-gly)$_2$</td>
<td>60</td>
</tr>
<tr>
<td>BSP-GSH</td>
<td>6</td>
</tr>
<tr>
<td>BSP-cys-gly</td>
<td>25</td>
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**Fig. 3.** Representative chromatograms of HPLC analysis with post-column alkalinization and spectrophotometric detection at 578 nm of perfusate samples (A, B and C) and urine samples (D, E and F) at the end of the perfusion. Per fusate contained 2.5% pluronic F108 (A and D), 2.2% pluronic F108 + 2.0% bovine serum albumin (B and E) or 2.5% pluronic F108 + 1.0 mM acivicin (C and F). The compounds present were identified as BSP-(GSH)$_2$ (1), BSP-(cys-gly)$_2$ (2), BSP-GSH (3) and BSP-cys-gly (4).
Renal Disposition of BSP-GSH

The urinary excretion of BSP-GSH is somewhat lowered by addition of acivicin, which suggests that the rapid decline of the BSP-GSH concentration in the absence of acivicin was mostly due to conversion of BSP-GSH to BSP-cys-gly.

The experimental groups and the corresponding symbols are the same as those in figure 1.

References


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