Journal of Chromatography, 620 (1993) 121–128
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7052

High-performance liquid chromatographic determination of the *S*- and *R*-diastereoisomers of L-buthionine (*SR*)-sulfoximine in human plasma and urine

James M. Brennan*, Peter J. O'Dwyer and Robert F. Ozols

Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111 (USA)

Frank P. LaCreta☆

Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111 (USA)

(First received February 15th, 1993; revised manuscript received June 18th, 1993)

ABSTRACT

A sensitive and reproducible HPLC procedure was developed for the simultaneous determination of the diastereoisomers of the synthetic amino acid L-buthionine-(*SR*)-sulfoximine (BSO) in human plasma and urine. Plasma samples were prepared for analysis by addition of internal standard (L-norleucine) followed by ultrafiltration using disposable centrifugal filtration units. Urine samples received internal standard followed by solid phase extraction using disposable C_{18} cartridges. All samples were derivatized with phenylisothiocyanate (PITC). The derivatized amino acids were separated by HPLC on an octyldecyl column (250 mm × 4.6 mm I.D., 5 μm particle size) using a mobile phase of sodium acetate–acetonitrile–triethylamine–ethylaminediaminetetraacetic acid. The column effluent was monitored at 254 nm and quantitation was performed using peak areas. The linear range for each diastereoisomer of L-(*SR*)-BSO was from 2 to 100 μg/ml in plasma and from 10 to 1000 μg/ml in urine. The method is reproducible, convenient and sensitive, illustrating its utility for application in pharmacokinetic studies.

INTRODUCTION

Buthionine sulfoximine (*S*-*n*-butyl homocysteine sulfoximine; BSO), synthesized by Griffith and Meister in 1979, is a potent and selective inhibitor of γ-glutamylcysteine synthetase [1]. Inhibition of this enzyme causes depletion of intracellular glutathione (GSH), a cysteine containing tripeptide with many metabolic functions [2]. Recently, it was shown that elevated intracellular GSH levels are related to acquired resistance to alkylating agents and cisplatin [3,4]. *In vitro* [5] and *in vivo* [6] studies showed that GSH depletion occurred following BSO exposure, resulting in sensitization to chemotherapeutic agents.

A previous study on the pharmacokinetics of BSO in mice was performed by Smith *et al.* [7]. Because the BSO used is a racemic mixture of the *R*- and *S*-diastereoisomers of L-BSO, and the analytical method did not quantitate the isomers separately, the interpretation of the diastereoisomers is necessarily incomplete. The analytical method used in that study was originally described by Duff and Murrill [8]. Because purified *R*- and *S*-isomers were not available, the separate diastereoisomers were not quantitated. Other factors limited the method. Most importantly,

* Corresponding author.
☆ Present address: Bristol-Myers Squibb, Department of Metabolism and Pharmacokinetics, Syracuse, NY 13221, USA.

0378-4347/93/$06.00

each sample was prepared immediately prior to injection onto the HPLC column because of the instability of the *o*-phthalaldehyde derivative. Additionally the internal standard, butyrophenone, required a fluorescence detector in addition to the UV detector used for the *o*-phthalaldehyde derivative. While the authors were able to achieve baseline separation of the BSO diastereoisomers, they did report that the changes in chromatographic parameters necessary to attain separation resulted in unacceptably long (>75 min/sample) analysis times.

Recently, Campbell *et al.*, showed that L-(*S*)-BSO is a more potent inhibitor of glutamylcysteine sythetase than is L-(*R*)-BSO [9], illustrating that an analytical method capable of quantitating the individual isomers is essential. In the same reference, a method for the analytical and preparative separation of the *R*- and *S*-isomers of BSO was described [9]. That method used chiral solvent reversed-phase HPLC and post-column derivatization with *o*-phthalaldehyde. However, the authors utilized the procedure to isolate the BSO isomers for biochemical studies, and therefore did not describe accuracy, precision or detection limit of their method.

To determine the pharmacokinetics of BSO in subjects from a Phase I clinical trial, we wanted an analytical method that would allow quantitation of the individual *R*- and *S*-isomer, and enable the use of an autosampler. *o*-Phthalaldehyde, one of the commonly used amino acid derivatization reagents, produces a derivative that is unstable at room temperature. Therefore, a reagent that produced a stable derivative was necessary.

A reagent commonly used in the analysis of amino acids, phenylisothiocyanate (PITC), has many of the qualities that are desirable for this application. Known as the Edman reagent, PITC reacts readily with primary and secondary amino acids, producing a stable derivative that strongly absorbs UV light at 254 nm. Its use for the pre-column derivatization of amino acids was originally described by Heinrikson and Meredith [10]. Recently, a simple derivatization procedure for the determination of free amino acids in plasma was reported [11]. Using ultrafiltration, a single-step sample preparation procedure was described that yielded results comparable to deproteinization with sulphosalicylic acid prior to PITC derivatization.

In this reported we describe a reversed-phase HPLC method for the determination of the *R*- and *S*-isomers of L-BSO. This method uses the single-step ultrafiltration and sample preparation procedure for plasma as described above for the pre-column derivatization of amino acids with PITC. Urine samples are prepared for derivatization by extraction with C_{18} Bond Elut column as described previousely [12]. Reversed-phase HPLC and UV detection are used to quantitate BSO isomers in human plasma and urine samples.

EXPERIMENTAL

Chemicals

The purified *S*- and *R*-diastereoisomers of L-BSO (*S*-BSO and *R*-BSO) were a generous gift of Owen Griffith (Cornell University Medical College, New York, NY, USA). EDTA, L-norleucine, L-serine and triethylamine were purchased from Sigma (St. Louis, MO, USA). Sodium acetate, trihydrate was from J. T. Baker (Phillipsburg, NJ, USA). The chromatography solvent acetonitrile was purchased from Burdick and Jackson (Muskegon, MI, USA). High-purity water was produced with a Nanopure ultrapure water system (Barnstead, Dubuque, IA, USA). Control plasma was obtained from Interstate Blood Bank (Philadelphia, PA, USA). Control urine was obtained from a normal male subject.

Sample preparation

Plasma samples (200 μl) were prepared for ultrafiltration by addition of 200 μl of 0.1 *M* HCl for blank samples, or the internal standard (L-norleucine, 100 μg/ml) dissolved in 0.1 *M* HCl, for analytical standards, quality controls or patient samples. A standard curve was prepared by adding 20 μl of the appropriate *R*-BSO and *S*-BSO standard solution to 460 μl of control plasma. After vortex-mixing, 200 μl of each plasma

standard was combined with 200 μl of internal standard solution. All samples were vortex-mixed and transferred to 10 000 nominal molecular weight limit (NMWL) ultrafiltration units (Millipore, Bedford, MA, USA) and centrifuged at 5000 *g* for 15 min. A 40-μl aliquot of the ultrafiltrate was derivatized by addition of 80-μl of a freshly prepared solution containing ethanol (100%), PITC and triethylamine (40:1:1), in 10 × 75 mm borosilicate culture tubes. After vortex-mixting, the samples were allowed to react for 15 min at room temperature, after which 10 μl of L-serine (316 mg/ml) was added to each sample to consume any unreacted PITC. After vortex-mixing the samples were allowed to react for an additional 15 min, then evaporated to dryness under vacuum. The residue was resuspended in 600 μl of eluent A (composition described below).

Urine samples (100 μl) were prepared for extraction by addition of either 100 μl of 0.1 *M* HCl for the blank or 100 μl of the internal standard. The standard curve was prepared by adding 40 μl of each of the appropriate *R*-BSO and *S*-BSO standard solutions to 20 μl of control urine. After vortex-mixing, 100 μl of internal standard was added. A 200-μl aliquot of the resulting solution was then loaded on a preconditioned (3 ml methanol followed by 3 ml of water) C_{18} Bond Elut disposable cartridge (Varian, Harbor City, CA, USA). Elution with 300 μl of 0.1 *M* HCl was followed by 500 μl of 30% acetonitrile in 0.1 *M* HCl. All eluates were collected (1.0 ml total volume) and thoroughly vortex-mixed. A 40-μl aliquot was removed and derivatized by the procedure used for plasma samples.

Recovery of the BSO isomers and norleucine from plasma and urine was determined. Two sets of appropriate standards ($n = 6$) in water and one set of standards in either plasma (1 to 100 μg/ml) or urine (10 to 1000 μg/ml) were prepared. One set of standards in water was derivatized and analyzed directly and the second was handled according to the procedure for the corresponding matrix (ultrafiltration for plasma, C_{18} Bond Elut extraction for urine). The standards in plasma and urine were processed as described. The slopes, intercepts, and correlation coefficients of the standard curves prepared in water, plasma and urine were calculated and compared. Percent recovery was calculated from:

$$\text{Percent recovery} = \frac{\text{Slope of plasma or urine curve}}{\text{Slope of water curve}} \times 100$$

High-performance liquid chromatography

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP-1090 series A liquid chromatograph equipped with an autosampler/autoinjecter and a HP1040A diode-array UV detector. The injection volume was 250 μl and the column effluent was monitored at 254 nm (20 nm bandwidth). The detector range was 0.01 AUFS until 34 min when the range was increased to 0.05 AUFS. The chromatograph was operated with a Hewlett-Packard HP-85B personal computer and data were processed with a DPU multi-channel integrator. Chromatography was performed on an Adsorbosphere C_{18} reversed-phase analytical column, 5 μm 250 mm × 4.6 mm I.D. (Alltech Associates, Deerfield, IL, USA) preceded by a 10 mm × 4.6 mm, 5 μm Adsorbosphere C_{18} guard column. *S*-BSO and *R*-BSO were eluted, with retention times of 26.0 and 26.6 min, respectively, by an isocratic mobile phase at a flow rate of 1.25 ml/min. The binary mobile phase was composed of 91.5% eluent A and 8.5% eluent B. Eluent A was prepared from 0.14 *M* sodium acetate in 1 l of water containing 0.5 ml of TEA, 0.2 ml of EDTA (1 mg/ml), titrated to pH 6.40 with glacial acetic acid, and to 940 ml of this solution 60 ml of acetonitrile were added. Eluent B consisted of 60% acetonitrile in water containing 0.2 ml/l of EDTA (1 mg/ml). The internal standard, L-norleucine (retention time of 39.3 min), was eluted by a gradient mobile phase. Beginning at 30 min, a linear gradient was introduced, going from 8.5 to 45% eluent B in 10 min, then increasing to 100% B in 2 min. After remaining at 100% eluent B for 6 min, the mobile phase was returned to 91.5% eluent A over 2 min. The column was then reequilibrated for 8 min prior to the next injection.

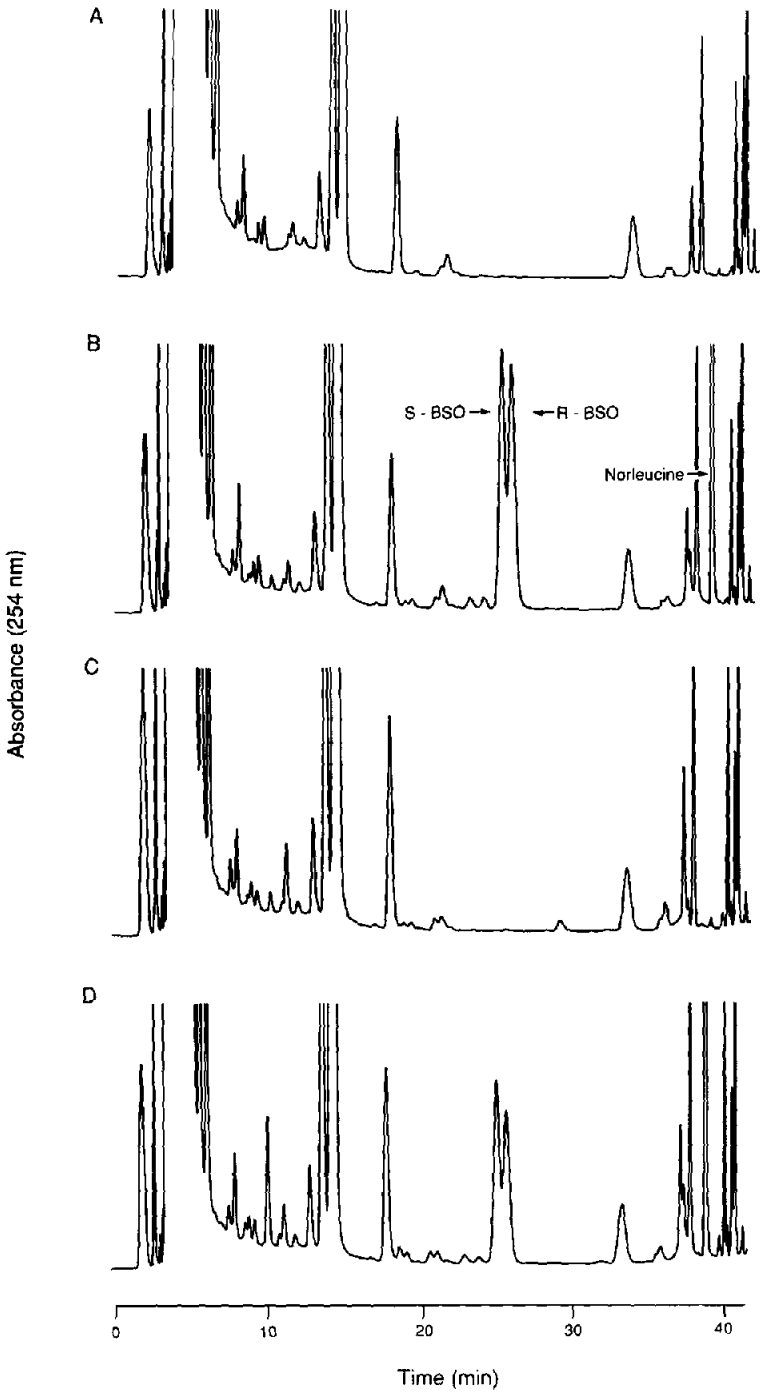

Fig. 1. Chromatograms of blank and L-BSO-containing plasma. (A) Control plasma; (B) control plasma spiked with *S*-BSO (100 μg/ml), *R*-BSO (100 μg/ml), and L-norleucine (50 μg/ml); (C) pretreatment patient plasma; (D) post-treatment patient plasma obtained two hours after the beginning of a 30-min intravenous infusion (BSO dose = 15 g), *S*-BSO = 76.2 μg/ml, *R*-BSO = 64.4 μg/ml and spiked with L-norleucine (50 μg/ml). For all chromatograms, 250 μg injected and detector at 0.05 AUFS.

RESULTS AND DISCUSSION

After filtration and derivatization, HPLC of control plasma (Fig. 1A) yielded a chromatogram clear of interfering peaks at the retention times of *S*-BSO, *R*-BSO and L-norleucine. The chromatogram of control plasma spiked with L-(*SR*)-BSO and L-norleucine (Fig. 1B) demonstrated the separation of the phenylthiocarbamyl amino acid derivatives.

The pre-treatment patient plasma was void of interfering peaks (Fig. 1C) and a post-treatment patient sample obtained 2 h after the end of drug infusion (Fig. 1D) yielded a plasma concentration of 76.2 μg/ml of *S*-BSO and 64.4 μg/ml of *R*-BSO from an L-(*SR*)-BSO dose of 7.5 g/m^2. The concentrations of BSO isomers in patient samples obtained 8 h post-injection were greater than the lowest linear limit of 2 μg/ml of plasma; the samples obtained shortly after dosing required dilution.

The introduction of L-norleucine as an internal standard was done to improve the precision of the analysis. This primary amino acid was chosen because its retention time was in a clear area of the chromatogram, and it is not found *in vivo*.

There was a linear relationship between the peak-area of each BSO isomer to L-norleucine and the concentration of the individual diastereoisomer. The linear range was 2.0 to 100.0 μg/ml, with a lower limit of detection of 1.0 μg/ml. The minimal detectable concentration was determined from the L-(*SR*)-BSO peaks resulting in a signal-to-noise ratio of 2 and was calculated by direct measurement of blank and drug-containing plasma chromatograms obtained with detector set at 0.002 AUFS. The average equation obtained from *R*-BSO standard curves, prepared daily with patient samples, was $y = 0.0060x + 0.0003$ ($r = 0.9996$) with a coefficient of variation (C.V.) for the slope of 6.5%. The average equation obtained for *S*-BSO was $y = 0.0054x + 0.0011$ ($r = 0.9998$) with a C.V. of 6.0% for the slope.

The accuracy and precision of the plasma analytical method were evaluated using two different L-(*SR*)-BSO-containing plasma quality controls prepared separately and stored frozen at −80°C. Each quality control was prepared in duplicate and the results from six analyses are shown in Table I. The results indicate that the individual BSO isomers can be measured accurately and precisely in plasma.

The ratio of the slopes of the standard curves prepared and derivatized directly in water versus processed standards showed that the recovery of *S*- and *R*-BSO was 121% and 113%, respectively. When standards prepared in water were ultrafiltered prior to derivatization, percent recovery compared to plasma was 105% for *S*-BSO and 108% for *R*-BSO. When unfiltered standards in water were compared to filtered standards in water the percent recovery was 115% and 105% for *S*- and *R*-BSO, respectively. The mean value (± S.D., $n = 6$) for the peak area of the internal standard obtained from the standard curves

TABLE I

PLASMA INTER-DAY ASSAY VARIABILITY AND ACCURACY

Spiked concentration (μg/ml)	Found concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Error (%)	*n*
S-BSO				
94	93.1 ± 4.8	5.2	1.0	12
9.4	8.9 ± 0.4	4.5	5.3	12
R-BSO				
94	93.3 ± 4.9	5.2	0.7	12
9.4	8.8 ± 0.4	4.5	6.4	12

were: 1838 ± 74 for the unfiltered standards in water, 1732 ± 249 for the filtered standards in water, and 1861 ± 28 for the processed plasma standards. The norleucine peak-area values from the three sets of standard curves were not significantly different from each other by analysis of variance (ANOVA). The intercepts from each standard curve did not differ significantly from zero. These findings indicate that there is no binding of the isomers to the ultrafiltration membrane and that the recovery and reaction efficiency of the BSO isomers and the internal standard are comparable in plasma ultrafiltrate or water.

The urine chromatogram was similar in appearance to the plasma chromatogram. As seen in Fig. 2A, the pre-treatment patient urine was void of interfering peaks, and the first 3-h aliquot (Fig. 2B) had sufficient L-(*SR*)-BSO to require dilution before extraction and derivatization. The difference in appearance from one chromato-

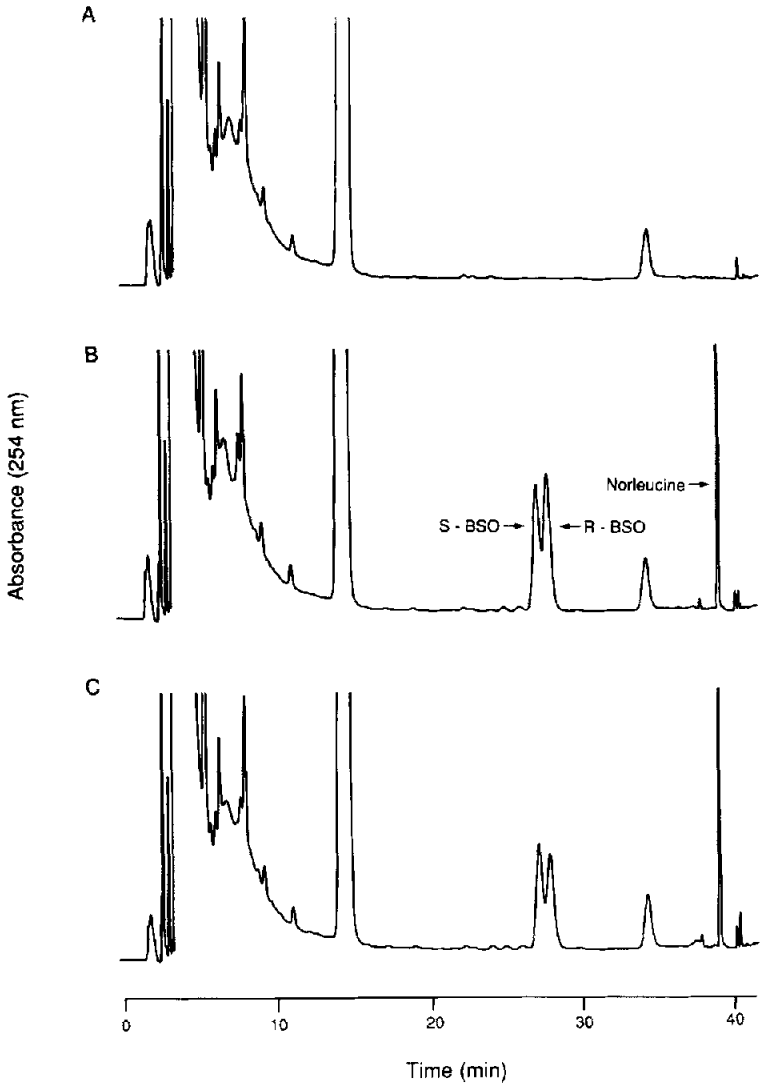

Fig. 2. Chromatograms of patient urine samples. (A) Pre-treatment patient urine from a total volume of 100 ml; (B) 0–3 h patient urine from a total volume of 1100 ml; diluted 1:19, *S*-BSO = 5.92 mg/ml, *R*-BSO = 6.64 mg/ml and spiked with L-norleucine (50 μg/ml); (C) 3–6 h patient urine from a total volume of 200 ml; diluted 1:19, *S*-BSO = 5.27 mg/ml, *R*-BSO = 4.82 mg/ml, and spiked with L-norleucine (50 μg/ml). For all chromatograms, 250 μl injected and detector at 0.05 AUFS.

TABLE II

URINE INTER-DAY ASSAY VARIABILITY AND ACCURACY

Spiked concentration (μg/ml)	Found concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Error (%)	*n*
S-BSO				
920	960.6 ± 54.2	5.6	4.4	17
92	105.2 ± 7.1	6.7	14	17
R-BSO				
920	914.5 ± 43.7	4.8	0.6	17
92	96.6 ± 8.1	8.4	5.0	17

gram to the other is due to changes in urine concentration from one sample collection period to another.

There was a linear relationship between the peak-area ratios of *S*-BSO and *R*-BSO to L-norleucine and the concentration of *S*-BSO and *R*-BSO from urine standards. The linear range was from 10.0 to 1000.0 μg/ml, with a lower limit of detection of 6.0 μg/ml). The average equation obtained from standard curves ($n = 9$), prepared daily with patient samples, was $y = 0.0054x + 0.0021$ ($r = 0.9990$) with a C.V. for the slope of 11.2% for *R*-BSO. The average equation for *S*-BSO, from the same standard curves, was $y = 0.0049x + 0.0020$ ($r = 0.9994$) with a C.V. of 8.8% for the slope.

The accuracy and precision of the urine analytical method were evaluated using two different L-(*SR*)-BSO-containing urine quality control standards, prepared separately and stored frozen at −80°C prior to analysis. Each quality control sample was run in duplicate along with patient samples on each day of analysis. The results given in Table II indicate that the method is accurate and reproducible.

Recovery of BSO from urine samples was investigated. Standard curves prepared in water, water extracted through C_{18} Bond Elut columns, and urine extracted through C_{18} Bond Elut columns were compared. Recovery of BSO isomers from urine compared to water extracted through a C_{18} Bond Elut column was 105% and 106% for *S*- and *R*-BSO, respectively. Recovery of BSO isomers from water following C_{18} extraction compared to water alone gave recoveries of 103% for *S*-BSO and 99% for *R*-BSO. The mean ± S.D. values for norleucine peak area from the three standard curves ($n = 6$) were: 260 ± 60 for water, 275 ± 57 for water extracted through a C_{18} column, and 289 ± 20 for standards prepared in urine. The values for internal-standard peak area for the three preparations were not different statistically. These results indicate quantitative recovery of the BSO isomers and norleucine from urine and C_{18} Bond Elut extraction columns.

One problem became apparent during the development and application of this analytical method. The column efficiency deteriorated gradually and limited the useful lifetime to approximately 120-sample analyses. The steady deterioration in column efficiency may be attributed to residual PITC in the analytical samples, which was not removed under vacuum. Our vacuum system had a maximum capacity of 50.0 Pa, well above the recommended 6.7–8.0 Pa needed to adequately remove excess PITC. The addition of L-serine, to consume excess derivatization reagent, reduced but did not eliminate unreacted PITC. Thus, the number of samples analyzed and the consistency of chromatographic variables (resolution, retention time, etc.) were monitored closely.

Baseline separation between the BSO isomers was not achieved using this gradient profile. Improved separation was obtained by decreasing

the solvent strength and prolonging the isocratic step of the elution profile. Because of the length of each analysis and the loss of sensitivity caused by band broadening (and the resultant decrease in peak height) with longer analysis times, little was gained by achieving baseline separation. Therefore, to assure that accurate peak-area measurements could be obtained the resolution (R_s) of the BSO peaks was the chromatographic parameter considered most important to monitor. For this purpose R_s values of 1 or greater were considered adequate to accurately quantitate the individual peaks. It has been shown that for two chromatographic peaks of equal heights with an R_s value of 1, there is no error in the area measurement [13]. The heights of the BSO peaks were usually equal and never varied by a ratio greater than 3 to 1. Therefore, the error associated with the integration of the minor band was always less than 3%, and is acceptable for this application. R_s was calculated from:

$$R_s = 1.176 \times \frac{t_{rR\text{-BSO}} - t_{rS\text{-BSO}}}{t_{w1/2R\text{-BSO}} + t_{w1/2S\text{-BSO}}}$$

where t_r is the retention time and $t_{w1/2}$ is the peak-width at half-height of the corresponding BSO chromatographic peak. For a new column, R_s values of 1.25 were routinely obtained. When R_s values decreased below 1, a new column was used.

Application of the analytical method to the analysis of serum samples (obtained from patients in our Phase I study of BSO in combination with melphalan) is illustrated in Fig. 1D. Following a 30-min infusion of a 7.5-g/m^2 dose of BSO the disappearance of *R*- and *S*-BSO isomers from plasma was similar. Analysis of the dosing solution revealed that the clinical preparation of BSO consisted of 54% *R*- and 46% *S*-BSO. The BSO was dosed on a total g/m^2 basis. For the 7.5-g/m^2 dose for the patient in Fig. 1D, doses of *S*-BSO of 3.4 g/m^2 and *R*-BSO of 4.1 g/m^2 would result. The difference in dose administered is illustrated by the greater initial concentrations of *R*-BSO compared to *S*-BSO. However, the plasma disappearance of *R*-BSO was faster than that of *S*-BSO. Analysis of urinary excretion of BSO showed that the renal clearance of both isomers was about 100 ml/min. However, non-renal clearance of *R*-BSO was approximately 50% greater than that of *S*-BSO, suggesting a metabolic component of BSO elimination with stereoselectivity for the *R*-BSO. Further studies on the metabolism of the BSO isomers will be facilitated by this analytical method.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the expert secretarial assistance of Catherine Thompson.

REFERENCES

1 O. W. Griffith and A. Meister, *J. Biol. Chem.*, 254 (1979) 7558.
2 A. Meister and M. E. Anderson, *Ann. Rev. Biochem.*, 52 (1983) 711.
3 K. Suzukake, B. P. Vistica and D. T. Vistica, *Biochem. Pharmacol.*, 32 (1983) 165.
4 T. C. Hamilton, R. C. Young and R. F. Ozols, *Semin. Oncol.*, 11 (1984) 285.
5 T. C. Hamilton, M. A. Winkler, K. G. Louie, G. Batist, B. C. Behrens, K. R. Grotzinger, W. M. McKay, R. C. Young and R. F. Ozols, *Biochem. Pharmacol.*, 34 (1985) 2583.
6 R. F. Ozols, K. G. Louie, J. Plowman, B. C. Behrens, R. L. Fine, D. Dykes and T. C. Hamilton, *Biochem. Pharmacol.*, 36 (1987) 147.
7 A. C. Smith, J. T. F. Liao, J. G. Page, M. G. Wientges and C. K. Grieshaber, *Cancer Res.*, 49 (1989) 5385.
8 R. Duff and E. Murrill, *J. Chromatogr.*, 385 (1987) 275.
9 E. B. Campbell, M. L. Hayward and O. W. Griffith, *Anal. Biochem.*, 194 (1991) 268.
10 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
11 B. L. Rosenlund, *J. Chromatogr.*, 529 (1990) 528.
12 S. A. Cohen, M. Meys and T. L. Tarvin, *The Pico-Taq Method: A Manual of Advanced Techniques for Amino Acid Analysis*, Millipore, Bedford, MA, 1989, pp. 58–62.
13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd ed., John Wiley and Sons, New York, 1979, pp. 45–48.