Journal of Chromatography, 385 (1987) 275–282
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1062

DETERMINATION OF L-BUTHIONINE-(*S*,*R*)-SULFOXIMINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH *o*-PHTHALALDEHYDE DERIVATIZATION AND FLUOROMETRIC DETECTION

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SUMMARY

The development of a high-performance liquid chromatography system for the analysis of L-buthionine-(*S*,*R*)-sulfoximine (BSO) in human plasma is described. *o*-Phthalaldehyde derivatization and fluorescence detection were used. The *R*- and *S*-BSO peaks were partially separated from each other and completely separated from the matrix components. The limit of detection for BSO was 2 μg/ml plasma.

INTRODUCTION

L-Buthionine-(*S*,*R*)-sulfoximine (BSO), a potent and specific inhibitor of glutathione synthesis[1–3], is being considered as a possible sensitizer for chemotherapeutic agents[4–10]. In order to perform pharmacokinetic and toxicological studies on this drug, the National Cancer Institute required an analytical method capable of detecting μg/ml levels of BSO in small quantities of mouse and human plasma, as well as in *in vitro* growth media[11]. No specific methods for determining BSO in biological fluids have been published; the method described below was therefore developed for this purpose.

The method developed also enabled partial resolution and separate quantitation of the *R*- and *S*-isomers of BSO (after derivatization with *o*-phthalaldehyde) on a C_{18} column with an ion-pairing mobile phase. The use of these relatively inexpensive reagents and columns, in combination with the proven durability record of C_{18} columns, make this system suitable for use with complex biological matrices. All previously published methods for the separation of the optical isomers of sulfoximines by high-performance liquid chromatography (HPLC) have used chiral stationary phases[12,13].

EXPERIMENTAL

Materials

The test compound (BSO, Lot No. 85-06-17-01) was supplied by Monsanto

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(St. Louis, MO, U.S.A.) under a contract with the National Cancer Institute. The identity and purity of the test sample were determined by spectroscopic [IR, UV, NMR, and mass spectrometry (MS)], titration, and chromatographic (HPLC and thin-layer chromatography) analyses. The chromatographic internal standard (I.S.), butyrophenone, was purchased from Eastman Kodak (Rochester, NY, U.S.A.). The sample solvent was HPLC grade Milli-Q (Millipore, Bedford, MA, U.S.A.) filtered water. HPLC-grade methanol from Burdick & Jackson (Muskegon, MI, U.S.A.) was used as the solvent for the I.S., the protein precipitating agent for plasma sample preparation, and the solvent for the derivatizing reagent. The derivatizing reagent contained *o*-phthalaldehyde (Pierce, Rockford, IL, U.S.A.), sodium borate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and 2-mercaptoethanol (Sigma, St. Louis, MO, U.S.A.). Fresh frozen human plasma, containing anticoagulant CPDA-1, was purchased from the Community Blood Center (Kansas City, MO, U.S.A.) and frozen mouse plasma, containing 3–8% sodium citrate, from Pel-Freeze Biologicals (Rogers, AR, U.S.A.). The RPMI 1640 growth media were supplied by the National Cancer Institute.

Standard solutions

A stock BSO solution (2.940 mg/ml) was prepared by dissolving 73.50 mg of the drug in 25 ml of water. Serial dilutions (17:25, 2.5:10, 4:10, 5:10, and 2:10) were also made with water. The solutions from the last four serial dilutions (the working solutions) were used to spike plasma samples (0.5 ml), providing BSO concentrations of 99.95, 39.98, 19.99, and 3.998 μg/ml plasma. Solutions for interday analysis were similarly prepared to provide BSO concentrations of 98.10, 39.24, 19.62 and 3.924 μg/ml. A stock solution of I.S. (1.3 μl/ml) was prepared by diluting 130 μl of butyrophenone to 100 ml with methanol–water (50:50, v/v). An aliquot (100 μl) of this solution was added to the sample solutions to provide a final I.S. concentration in plasma of 0.26 μl/ml.

The derivatizing reagent was prepared by the method of Jones *et al.*[14]. *o*-Phthalaldehyde (200 mg) was dissolved in 5 ml of methanol in a 50-ml volumetric flask. A 200-μl aliquot of 2-mercaptoethanol was then added to the flask, and it was diluted to volume with 0.01 *M* sodium borate buffer (pH 9.5).

Chromatographic conditions

Two detectors were used for the analysis. One, detector A, was used to detect the derivatized BSO, the other, detector B, was used to detect the internal standard. Detector A was a Schoeffel Instrument (Westwood, NJ, U.S.A.) Model FS 970 L.C. The fluorometer was set at an excitation wavelength of 340 nm and equipped with a 470-nm emission cut-off filter. The sensitivity setting was 4.50 and the range was 0.1 μA. Detector B was a Waters Assoc. (Milford, MA, U.S.A.) Model 440 fixed-wavelength UV detector equipped with a 254-nm filter and set at an attenuation of 1.0 a.u.f.s. The two Waters Assoc. 6000A pumps were controlled by a Waters Assoc. Model 680 automated gradient controller. Mobile phase A was 0.005 *M* tetrabutylammonium hydroxide (adjusted to pH 7.4 with phosphoric acid), mobile phase B was methanol. The flow-rate was 1 ml/min. The gradient program was mobile phase A–mobile phase B (51:49) for 45 min, a 15-min linear gradient to A–B (30:70), a 10-min linear gradient to A–B (51:49), and A–B (51:49) for 5 min. The separation

was performed on an Altex (San Ramon, CA, U.S.A.) Ultrasphere C_{18} column of 5 μm particle size, 250 mm × 4.6 mm I.D. A Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) was used in the overfilled mode to produce precise injections from the 20-μl fixed loop. The chromatographic data were integrated by means of a Nelson Analytical (Cupertino, CA, U.S.A.) Model 4400 data system with Xtrachrom software.

Sample preparation procedures

For the lower range assays, plasma samples (0.5 ml) were placed in Pyrex culture tubes with PTFE-lined caps. The samples were spiked with aliquots (100 μl) of the BSO working solutions. An aliquot of methanol (2.0 ml) was then added and the samples were mixed for 30 s on a Vortex-Genie (Scientific Inc., Bohemia, NY, U.S.A.). After mixing, the samples were centrifuged for 5 min in an analytical centrifuge. A 1-ml aliquot of the supernatant solution was removed and added to 200 μl of derivatizing reagent. This solution was then vortex-mixed for 10 s and filtered through an unwetted Sep-Pak® C_{18} cartridge. A 500-μl aliquot of the filtered solution was added to 100 μl of I.S. solution and analyzed by HPLC. The HPLC injection was made exactly 5 min after the supernatant solution was added to the derivatizing reagent.

For the higher-range assays, an additional dilution step was added. A 1-ml aliquot of the supernatant was added to 9 ml of methanol–water (50:50, v/v). A volume of 1 ml of this solution was then added to the derivatizing reagent. The rest of the procedure remained unchanged.

RESULTS

The assay method was validated in a drug/plasma concentration range of 4–100 μg/ml. Representative chromatograms of plasma samples spiked with BSO at four concentrations within this range are contained in Fig. 1. A chromatogram of unspiked plasma is included in the figure to illustrate the selectivity of the method. The eluted BSO consists of two peaks, approximately 50% separated from each other. The BSO peaks are completely separated from fluorescent components in the derivatized plasma matrix and have a retention time of 30 min. The I.S. is completely separated from UV-absorbing components in the derivatized plasma matrix and is also eluted in a window free of fluorescing components (Fig. 2). The estimated limit of detection of the method is 2 μg/ml plasma.

The linearity of the assay was determined from data obtained from samples prepared at four concentrations within the validated range and analyzed on two separate days. The linear correlation coefficients were: day 1, 0.999; day 2, 0.999.

The precision and accuracy of the method was determined by the analysis of spiked plasma samples at four concentrations with thrree replicates at each concentration. These analyses were repeated on a second day to test the interday variability of the method. The data from these experiments are presented in Table I. Plasma samples spiked at higher concentrations (399.8 and 599.7 μg/ml) were also assayed with the samples of the second day. The results of these studies are contained in Table I.

The recovery of the drug in the supernatant was studied by comparison of the

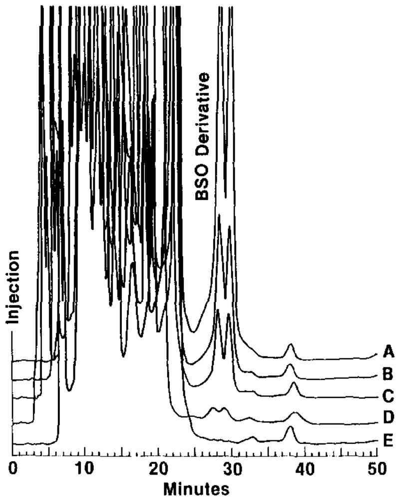

Fig. 1. Chromatograms of human plasma spiked with BSO at four levels and human plasma blank using a 25-cm, 5 μm, ultrasphere ODS column and the gradient mobile phase system described in *Chromatographic conditions*. A, 98.10 μl/ml plasma; B, 39.24; C, 19.62; D, 3.924; E, 0.

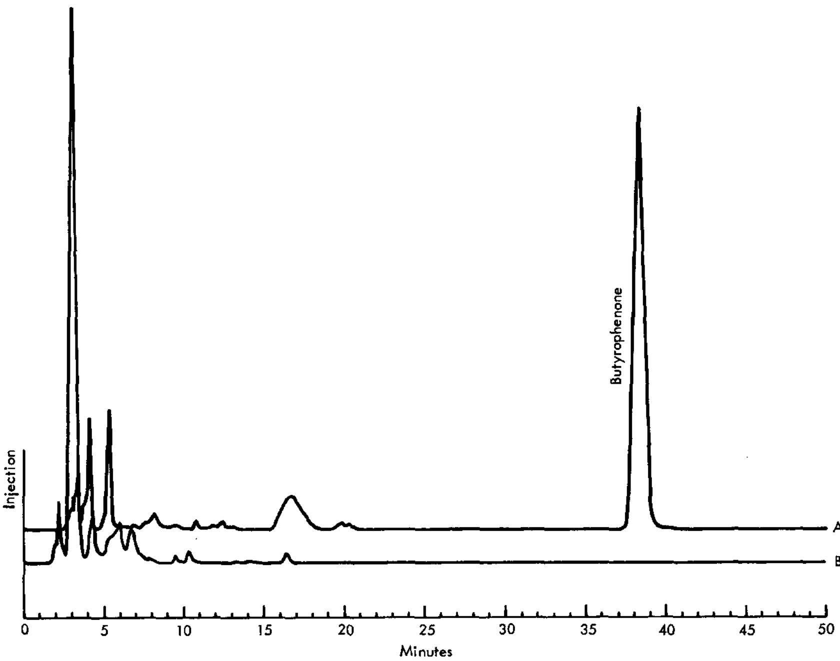

Fig. 2. (A) Human plasma spiked with butyrophenone (I.S.) at 0.26 μl/ml plasma detected at 254 nm UV. (B) Human plasma blank detected at 254 nm UV. For conditions see Fig. 1.

TABLE I

INTERDAY AND INTRADAY ASSAY VARIABILITY AND ACCURACY

$\text{Error} = \frac{\text{assay conc.} - \text{spiked conc.}}{\text{spiked conc.}} \times 100$. An F value of < 19.0 indicates no significant difference between the S.D.s of the results. A t value of < 2.78 indicates no significant difference between the means of the results.

Day 1						*Day 2*						*F*	*t*
Spiked concentration (μg/ml)	*n*	*Found (μg/ml)*		*C.V. (%)*	*Error (%)*	*Spiked concentration (μg/ml)*	*n*	*Found (μg/ml)*		*C.V. (%)*	*Error (%)*		
		Mean	*S.D.*					*Mean*	*S.D.*				
3.924	3	3.77	0.25	6.5	−3.9	3.998	3	4.66	0.21	4.5	16.7	1.4	4.8
19.62	3	19.9	0.66	3.3	1.4	19.99	3	20.6	1.5	7.3	2.8	5.0	0.7
39.24	3	39.1	0.78	2.0	−0.3	39.98	3	38.2	4.0	10.5	−4.6	26.4	—*
98.10	3	98.1	3.0	3.1	0.0	99.95	3	100.5	3.4	3.4	0.5	1.4	0.9
						399.8	2	410.9					
						599.7	2	628.7					

* t not calculated due to significant F value.

area ratios obtained on the spiked plasma samples during the accuracy and precision studies with those obtained on solution standards at the same concentrations and analyzed on the same day as the spiked plasma samples. The results of this study are shown in Table II. The recovery was independent of concentration, and there was no significant difference in the precision or in the results obtained on the two days.

TABLE II

PERCENTAGE RECOVERY FROM HUMAN PLASMA

Day 1		*Day 2*		*Recovery* (%)*		
Drug/plasma concentration (μg/ml)	*Recovery (%)*	*Drug/plasma concentration (μg/ml)*	*Recovery (%)*	*Mean*	*S.D.*	*R.S.D. (%)*
3.92	96.0	4.00	84.7	90.4	8.0	8.8
19.6	93.6	20.0	86.5	90.0	5.0	5.5
98.1	90.5	99.9	91.6	91.0	0.8	0.9
Mean =	93.4	Mean =	87.6			
S.D. =	2.7 (F = 1.78**)	S.D. =	3.6			
R.S.D. (%) =	2.9 (t = 2.23***)	R.S.D. (%) =	4.1			

Pooled estimate of recovery (%)
Mean = 90.5
S.D. = 4.2
R.S.D. (%) = 4.6
n = 6

* Recovery was independent of concentration.
** An F value < 19.0 indicates no significant difference between standard deviations of results.
*** An t value < 2.78 indicates no significant difference between means of results.

The pH of the tetrabutylammonium hydroxide portion of the mobile phase was adjusted to various levels between 5.0 and 7.4 with phosphoric acid. No significant change was observed in resolution between the two BSO peaks within this pH range.

By adjusting the solvent ratio of the mobile phase to A–B (53:47) the two BSO peaks were completely separated. However, this solvent ratio would give unacceptably long analysis times for the BSO in a plasma matrix (Fig. 3).

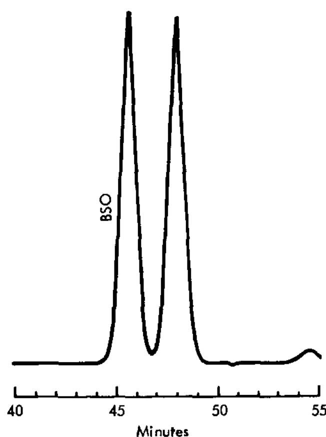

Fig. 3. BSO resolved by adjusting solvent ratio of mobile phase. Mobile phase: 0.005 *M* tetrabutylammonium hydroxide (adjusted to pH 7.4 with phosphoric acid)–methanol (53:47). Isocratic elution. Other conditions as in Fig. 1.

DISCUSSION

BSO has very limited solubility in non-aqueous solvents and also very weak absorption properties. The solubility properties of BSO precluded the use of simple extraction and cleanup techniques for increasing the concentration of the drug in the injected sample and removing extraneous matrix elements. HPLC was, therefore, used for chromatographic separation because this technique is tolerant of the multiple matrix elements present in protein precipitated filtrates of biological fluids. The weak absorption properties combined with the difficulties of concentrating the filtrates necessitated the production of a fluorescent derivative to lower the detection limit of the assay. Precolumn derivatization with *o*-phthalaldehyde was selected because it quickly and reproducibly formed a single unique reaction product with the primary amine that could be detected with good sensitivity[14–16].

The assay method developed is specific, allowing complete separation of the BSO from components in the matrix. It also gives adequate resolution between the two BSO peaks in an acceptable analysis time. The results obtained by varying the pH of the mobile phase and increasing the retention time of the BSO peaks indicate that these two peaks are the separated *R* and *S* isomers.

The partial separation of the two BSO peaks is adequate, assuming computer

integration is employed, for separate quantitation of the two peaks. Pharmacokinetic studies may not require this type of quantitation, but the separation would allow observation of possible selective metabolism of the isomers.

The inclusion of the Sep-Pak cleanup step in the method was necessary to remove interfering matrix elements. Sep-Pak filtration before derivatization was compared to that after derivatization. Only the Sep-pak filtration after derivatization was found to reduce the chromatographic area of the matrix components.

Numerous commercially available BSO analogues, non-endogenous amino acids, amine compounds, and compounds with native fluorescence (a total of 35) were chromatographed on the system in the search for an internal standard that would be detectable by fluorescence. None of these compounds were eluted in an optimum window, after the matrix components and BSO but under 40 min retention time. The selected I.S., butyrophenone, does meet these specifications. This I.S., however, had two limitations. It was not possible to add the I.S. prior to derivatization and, therefore, the I.S. serves only to monitor the chromatographic parameters of injection volume and column condition and not sample preparation. The I.S. also required separate ultraviolet detection. The precision and recovery data from this study indicated that there was not significant detector or sample preparation variability.

While the limit of detection of the method is somewhat higher than that obtained for plasma assays of compounds which can be extracted and concentrated, it is sufficient for the anticipated dosage levels. The loss of sensitivity can be attributed to sample dilution during the precipitation step, absence of BSO concentration, and the small injection volumes necessary to achieve separation from the matrix components.

An improvement on this method, although requiring additional instrumentation, would be automated precolumn derivatization. This would eliminate the necessity of manually derivatizing each sample prior to analysis.

ACKNOWLEDGMENT

The authors would like to thank the National Cancer Institute for funding Contract No. NO1-CM-37555.

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