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Quantitation of the diastereoisomers of L-buthionine-(*R*,*S*)-sulfoximine in human plasma: a validated assay by capillary electrophoresis

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Abstract

An assay for the diastereoisomers of the biochemical modifier L-buthionine-(*R*,*S*)-sulfoximine (BSO) in human plasma has been developed using capillary electrophoresis (CE). Separation of the diastereoisomers is achieved by the micellar electrokinetic chromatography (MEKC) mode of CE. Plasma is injected directly onto the separation capillary without any extraction step, and BSO is detected directly by ultraviolet absorbance measurements at 190 nm without prior derivatization. The whole assay, including capillary conditioning, takes approximately 30 min. Intra- and inter-day R.S.D. values are approximately 7% at sample concentrations around 25 μg ml^{-1}, and approximately 3% at sample concentrations around 500 μg ml^{-1}. The limit of detection in plasma is 3.9 μg ml^{-1} ($S/N = 2$). The assay has been used to quantitate the diastereoisomers of BSO in patient samples in a pharmacokinetic study.

1. Introduction

Buthionine sulfoximine (BSO) is an inhibitor of γ-glutamylcysteine synthetase, thus blocking glutathione biosynthesis [1]. BSO is a chiral compound with two chiral centres (structure shown in insert in Fig. 2), and is available as an investigational drug substance (Division of Cancer Treatment, National Cancer Institute, NSC-326231) which is an unequal mixture of L-buthionine-(*R*)-sulfoximine (L,*R*-BSO) and L-buthionine-(*S*)-sulfoximine (L,*S*-BSO). With any chiral drug there exists the possibility of stereoselectivity in its interactions with biological systems leading to pharmacological differences between the stereoisomers [2,3], and this is the case with BSO. It has been shown in vitro that L,*S*-BSO has a considerably greater activity in the inhibition of γ-glutamylcysteine synthetase than L,*R*-BSO, and in vivo L,*S*-BSO causes considerable glutathione depletion in mouse kidney, liver and pancreas while L,*R*-BSO has a minor effect on glutathione levels in the kidney only [4]. Depletion of cellular glutathione by BSO may modulate the toxicity of cancer chemotherapy and radiotherapy [5]. The diastereoisomeric L-(*R*,*S*)-BSO is now being used in clinical trials as a biochemical modulator in conjunction with radiotherapy for solid tumors. Clearly it is important to monitor the fate of both of the

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stereoisomers which are being administered and thus an assay suitable for the determination of the individual stereoisomers in biofluids is necessary.

There are a number of problems associated with the analysis of BSO in biological matrices. Firstly, BSO has a very low molar absorptivity, and the greatest UV absorption is at wavelengths below 200 nm. Therefore it is difficult to detect with HPLC using UV absorbance. Also, BSO is zwitterionic and highly polar, making extraction from biological matrices difficult. To overcome the difficulty of detection, the amine moiety of BSO can be derivatized with a fluorogenic or chromogenic reagent. Duff and Murrill [6] reported a HPLC method for the analysis of BSO in plasma using *o*-phthaldialdehyde (OPA) as a pre-column derivatization reagent. In this case, protein precipitation was performed before derivatization, and then further sample cleanup was achieved by passing the derivatized sample through a C_{18} solid-phase extraction cartridge to remove hydrophobic compounds. The derivatized BSO was unretained and collected in the cartridge eluent and then analyzed using a C_{18} column. Validation data were presented for total BSO measurements, since only incomplete resolution of the diastereoisomers was achieved under the validation conditions. There has been at least one published pharmacokinetic study in mice using this HPLC method, where partial resolution of the stereoisomers was shown and qualitative differences in the plasma clearance were noted, but the pharmacokinetic data were reported in terms of the total concentration of the mixture of the stereoisomers [7]. An analytical assay for the stereoisomers of BSO has been reported using ligand-exchange HPLC with cupric acetate and D-proline in the mobile phase, and a C_{18} stationary phase, with post-column OPA derivatization and fluorescence detection [4]. However, the use of this assay for determining BSO in plasma was not shown. Recently, a validated method for the determination of L,*R*- and L,*S*-BSO in human plasma and urine using HPLC has been published [8]. A plasma ultrafiltrate was reacted with phenylisothiocyanate, and the derivatized diastereoisomers were then separated by reversed-phase HPLC, with UV detection at 254 nm. We have also investigated the resolution of BSO stereoisomers using chiral stationary phases, but with unsatisfactory results [9].

Capillary electrophoresis (CE) methods harness electrical forces in capillary tubes to achieve separations based on electrophoretic or chromatographic parameters [10,11]. Numerous applications have been reported [12,13], including many in the field of biomedical analysis [14]. It is often possible to perform CE analyses of biofluids with minimal sample preparation. Each matrix presents its own particular difficulties. Urine is an extremely complicated mixture, and direct injection of urine samples puts great demands on the resolving power of the CE system [15]. Direct injection of plasma onto a capillary electroseparation system can be problematic because of the tendency of proteins to adhere to the fused-silica surface. For the monitoring of small drugs in biological matrices a simple solution to this problem is to use micellar electrokinetic capillary chromatography (MEKC) with sodium dodecyl sulphate (SDS) to complex the proteins, whereupon there is little tendency to stick to the capillary surface [16,17]. Such direct-injection methods can be useful for a variety of analytes using fairly standardized separation conditions [18].

To our knowledge, direct resolution of underivatized L-(*R*,*S*)-BSO has not been achieved by HPLC without the use of a chiral selector in either the mobile or stationary phase. Here, we report an assay using MEKC which allows the quantitation of L,*R*- and L,*S*-BSO in aqueous solution or in human plasma with direct sample injection. A brief summary of this work has previously appeared elsewhere [19]. There is no chiral selector in the buffer system, and separation is based simply on partitioning between the hydrophobic micellar pseudo-stationary phase and an aqueous buffer. The high separation efficiency of the CE system allows baseline resolution of the diastereoisomers despite very small differences in their partition coefficients. A considerable advantage of the CE method is that derivatization is not needed for detection. This is

because in CE the short detection pathlength allows measurements to be made at very low wavelengths despite buffer absorbance [20]. Thus BSO may be detected by UV absorption measurements at 190 nm.

2. Experimental

2.1. Apparatus

CE separations were carried out using either Applied Biosystems (Foster City, CA, USA) 270A or 270A-HT integrated CE systems. When using the 270A-HT, a circulating water bath set at 8°C was used to keep the sample compartment cool (lower temperatures caused precipitation of the SDS in the buffer vials). Capillaries of 375 μm O.D., and 50 or 75 μm I.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA) and cut into the desired lengths. The polyamide coating on the capillary was removed 22 cm from one end to provide a detection window. Injections were made by applying a vacuum (17 kPa) to the anodic end of the capillary. The capillary oven temperature was set to 30°C. Detection was by on-capillary UV absorbance at a wavelength of 190 nm with a risetime of 0.5 s. Data were analyzed using a Spectra-Physics (San Jose, CA, USA) Datajet integrator, and stored on a personal computer running Spectra-Physics Winner System software. Between runs the capillary was washed with the separation buffer, or with 0.1 *M* NaOH followed by buffer. When long automated sequences of samples were being run, different wash and run buffer reservoirs were used to avoid lowering of the level in the run buffer vial and consequent hydrodynamic flow. For the method validation, separations were performed at a field strength of 236 V cm^{-1} which resulted in a current flow of 32 μA in a 72 cm long, 50 μm I.D. capillary, and a 5-s injection time.

2.2. Chemicals

For analytical method development, L-(*R*,*S*)-BSO was obtained from Sigma (St. Louis, MO, USA). Aqueous standard solutions of L-(*R*,*S*)-BSO (10 mg ml^{-1}) were made using this material, and diluted appropriately in water to prepare calibration curves, or added to plasma for the validation studies. Patients were treated with L-(*R*,*S*)-BSO obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA (BSO injection, investigational dosage form, NSC-326231). It should be noted that these are both unequal mixtures of the BSO diastereoisomers. Samples of the individual stereoisomers were a gift from Dr. O.W. Griffith, Cornell University Medical College. Acetaminophen (4-acetamidophenol, used as an internal standard) was obtained from Aldrich (Milwaukee, WI, USA). Pooled human plasma was obtained from a local hospital, and kept frozen at −20°C until use.

The electrophoretic separations were performed using phosphate buffers with SDS as a surfactant additive. Analytical grade disodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from BDH (Toronto, Canada), while SDS was obtained from Aldrich or Sigma. The SDS should be free of UV absorbing impurities, and microbiological or similar grade is recommended. The absorption coefficient of SDS solutions should be measured before use, and for optimum sensitivity should be below ca. 2 M^{-1} at 200 nm. For the validation, 20 m*M* solutions of each phosphate were made, and mixed to give a pH of 6.8. SDS was then dissolved in the buffer solution to give a final SDS concentration of 170 m*M*. Buffers were filtered through a 0.45- μm membrane filter and degassed by ultrasonification before use.

2.3. Samples

The dose levels in this study ranged from 1.5 g m^{-2} to 5 g m^{-2}. BSO was administered intravenously over a period of 10 min. Blood was collected into iced Vacutainer EDTA tubes, through an indwelling catheter in the arm opposite the site of injection. Blood samples were immediately centrifuged, and the plasma was then stored at −80°C until analysis.

2.4. Validation procedure

The validation procedure was as follows. (1) A calibration curve was made over the concentration range of interest, from 2.5 μg ml^{-1} to 500 μg ml^{-1} for each diastereoisomer in plasma. (2) Ten samples at a high concentration (453.1 μg ml^{-1} L,*R*-BSO, 546.9 μg ml^{-1} L,*S*-BSO) and ten samples at a low concentration (22.7 μg ml^{-1} L,*R*-BSO, 27.3 μg ml^{-1} L,*S*-BSO) within the range of the calibration curve were analyzed. (3) Fifteen samples each at the same high and low concentrations were analyzed over a period of five days. Two determinations were made on each sample.

3. Results and discussion

The effect of buffer pH and SDS concentration on the separation of the BSO diastereoisomers was investigated to determine optimum separation conditions. Without SDS in the run buffer there was no measurable separation of the BSO diastereoisomers. Measurements of the effective mobility of BSO at pH 7.5, 8 and 8.5 indicate that BSO becomes increasingly negatively charged, going from pH 7.5 to 8.5, and that the p*I* for this compound is approximately 7.2. Baseline separation of the BSO diastereoisomers was achieved at SDS concentrations above ca. 50 m*M* with phosphate buffer systems at a variety of pH values from 6 to 8.5. There was no separation when SDS was replaced by 170 m*M* hexanesulphonic acid, a non-micelle-forming surfactant. This suggests that separation is indeed by micellar inclusion rather than by an ion-pairing effect or by an association between the hydrophobic tail of the surfactant and the alkyl chain of BSO. The effect of SDS concentration on the separation was investigated over a range of 0 to 190 m*M* SDS, using a 20 m*M*, pH 8 phosphate buffer. Over this concentration range the addition of SDS caused slight changes in electroosmotic flow, with the electroosmotic flow mobility varying from $6.36 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} with no added SDS to $5.45 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} with 190 m*M* SDS. Increasing SDS concentration lead to slower separations with increasing resolution. With concentrations of surfactant greater than 150 m*M* resolution was such that large injections (5 s, approximately 18 nl on a 72 cm long, 50 μm I.D. capillary) could be loaded onto the capillary to increase sensitivity whilst still maintaining baseline resolution.

The effect of pH on the effective mobility of BSO in buffers containing 150 m*M* SDS was investigated over a pH range of 6–8.5. These data are shown in Fig. 1. There is a minimum in the BSO effective mobility at pH 7. The maximum mobility difference between the stereoisomers was observed at pH 6, and the mobility difference reduced with increasing pH. This behaviour may be explained by considering the charge on the BSO molecule over this pH range. At higher pH values BSO becomes negatively charged, and so moves with its own electrophoretic mobility in the same direction as the SDS micelles against the electroosmotic flow. This effect is apparently greater than any reduction in partitioning into the micellar phase caused by increased electrostatic repulsion. As the pH is lowered below 7.2, BSO becomes positively charged, leading to increased interactions with the micelle. This hypothesis is supported by the observed decrease in mobility difference between the diastereoisomers with increasing pH, indicating a reduction in interactions with the SDS. Based on this information operation around pH 7 seems optimum, since migration time is minimised, while interaction of BSO with the micelles is relatively strong as evidenced by the mobility difference data. Operation at pH 6 was not favoured since peak shapes were poorer (tailing), and electroosmosis may be less stable at lower pH values [21].

The analysis of BSO in human plasma was investigated, the separation conditions being based on the above findings. A solution of 170 m*M* SDS in a 20 m*M*, pH 6.8 phosphate buffer was found to be suitable. The plasma underwent no pre-treatment except filtration through a 0.45-μm membrane filter to remove particulates which might otherwise block the capillary. Direct injection CE assays with SDS containing buffers have been shown to measure the total drug

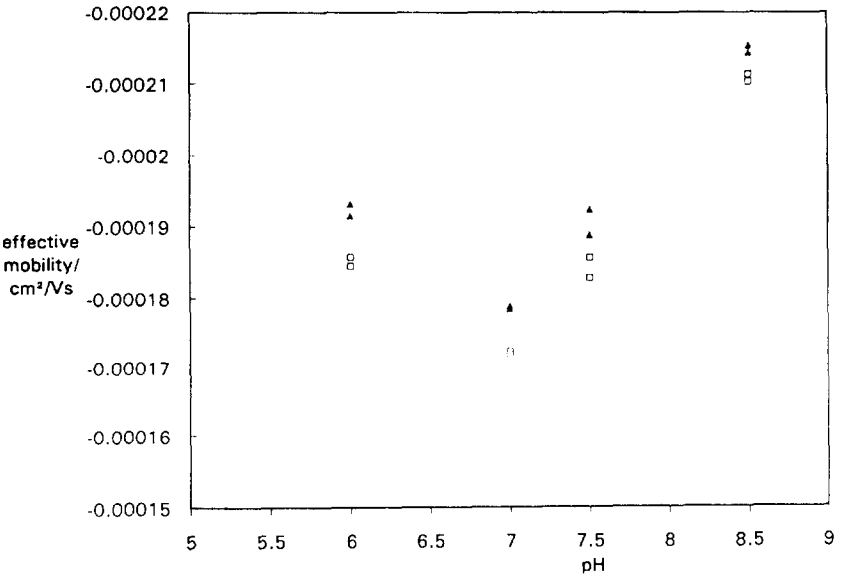

Fig. 1. Variation of BSO effective mobility as a function of buffer pH. ▲ = L,*R*-BSO; □ = L,*S*-BSO. Duplicate measurements were made at each pH.

concentration, since even highly protein-bound analytes are released by the denaturing action of the surfactant [22]. We confirmed that the response for BSO standards in plasma or water were the same. A typical separation of BSO spiked into human plasma is shown in Fig. 2

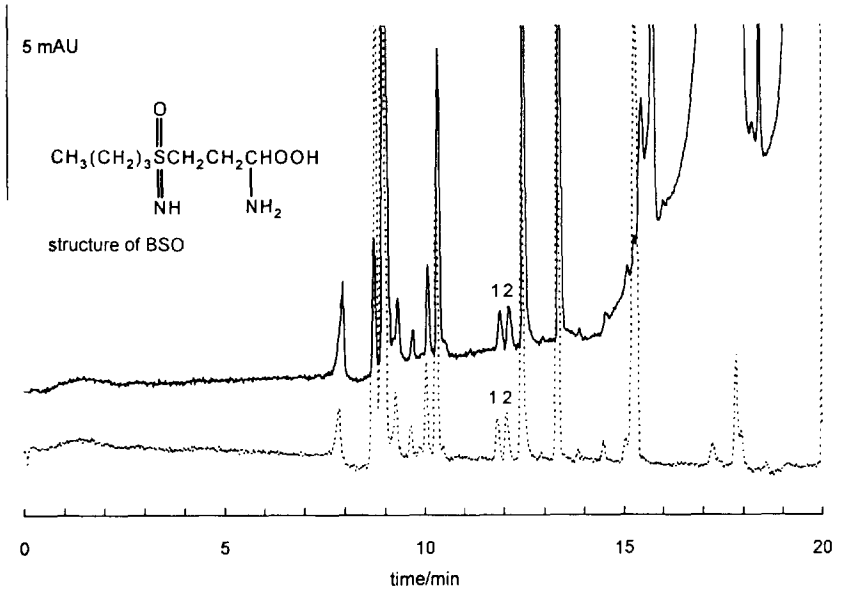

Fig. 2. Electropherograms of L,*R*-BSO (1) and L,*S*-BSO (2) (approximately 25 μg ml^{-1} each stereoisomer) analyzed in pooled plasma (upper trace) and ultrafiltered pooled plasma (lower trace). The plasma proteins which migrate after about 15 min are not apparent in the electropherogram of the ultrafiltered sample, but otherwise the two separations are very similar. Insert: structure of BSO.

(upper trace). Plasma proteins migrate after BSO. The effectiveness of the SDS in solubilising the plasma proteins is illustrated in Fig. 2 (lower trace), which is a subsequent electropherogram of the same sample, except that the sample has been ultrafiltered to remove proteins. The BSO migration times remain the same; peak areas are also not significantly changed, indicating that there is minimal protein binding of BSO in plasma.

With CE, detection can readily be performed at low-UV wavelengths, and this is necessary to achieve adequate sensitivity for BSO which has a UV absorbance which increases rapidly with decreasing wavelength below 210 nm. The lowest limits of detection for BSO using the present equipment were obtained at the lowest available wavelength of 190 nm. Cleanliness of the capillary window, detector focusing lens and in particular the light output of the lamp had a considerable impact on the limit of detection which could be achieved. A limit of detection of 2.3 μg ml^{-1} ($S/N = 2$) was obtained under favourable circumstances (new lamp, aqueous standard samples). However, during the assay validation the limit of detection was found to be 3.9 μg ml^{-1} ($S/N = 2$) for each diastereoisomer and this is more typical of what can be routinely achieved. Use of a larger I.D. capillary (75 μm) was also investigated, but did not lead to an improvement in sensitivity. Although CE measurements can be made using buffers which give rise to relatively high background absorbances [20], to obtain adequate sensitivity for the BSO assay we found that it is important to try and minimise the background absorbance. At 200 nm and below there is a significant absorbance due to the phosphate buffer itself, but the SDS makes the most significant contribution to the total absorbance. Major differences in low-wavelength absorbance were seen between different grades of SDS and even between different lots of SDS from the same manufacturers. 99% pure SDS (product L-4509) from Sigma had a measured absorbance coefficient of 13 M^{-1} at 200 nm, while molecular biology grade SDS (approximately 99% pure, product L-4390) from the same manufacturer was found to have an absorbance coefficient of only ca. 1 M^{-1}. In use in the CE system the less pure grade of SDS gave rise to considerably increased baseline noise, as shown in Fig. 3.

Migration time and mobility variability were determined over a number of runs. Initially there was a slow drift in migration time from around 10 min to 11.5 min over 20 consecutive runs made during the intra-day validation. This was measured in a capillary which had been previous-

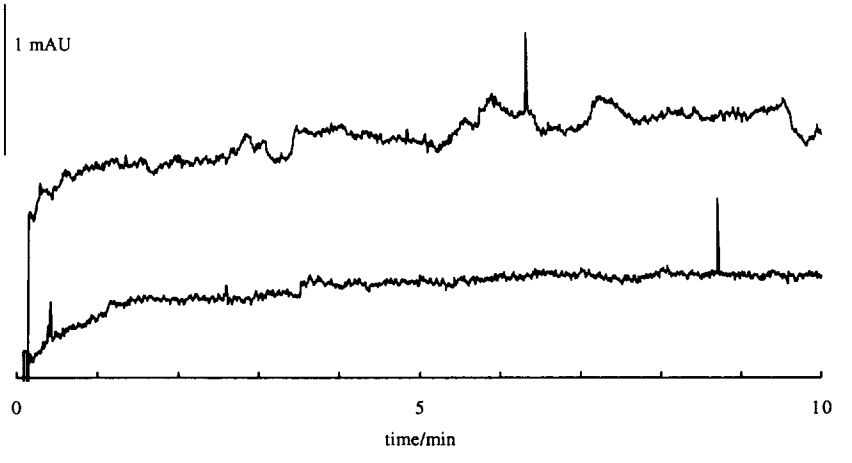

Fig. 3. Baseline noise at a detection wavelength of 190 nm recorded while current was flowing through the capillary using run buffers containing different grades of SDS. Upper trace, "99% pure", lower trace, "molecular biology grade". Run conditions as for validation separations (see text).

ly conditioned and used with aqueous BSO standard samples, but had never previously been used for the analysis of plasma samples. However, the effective mobility of each BSO stereoisomer for the same set of data was found to be quite constant. The R.S.D. for the mobility data was 0.45% for each diastereoisomer. During this sequence of injections the contents of the inlet and outlet buffer reservoirs were mixed to avoid any effects due to variation in the buffer composition, or changes in buffer level. Therefore, it seems most likely that the changes are due to a gradual alteration of the capillary surface. This may be due to a small degree of protein adhesion occurring on a capillary when it is first used for direct injections of plasma despite the presence of SDS in the buffer. The results for the inter-day validation which were performed afterwards on the same capillary did not show a systematic drift in migration times. These stabilised around 11.5 to 12 min, although the variability in mobility was still consistently lower than the variability in migration time. For the validation we did not perform an alkali wash to aggressively clean the capillary between runs, only a wash with the running buffer. We have observed that SDS-containing solutions are as effective as the commonly used 0.1 *M* NaOH wash in removing protein contaminants from capillary surfaces [23].

Unlike enantiomers, diastereoisomers have different physical properties including different molar absorption coefficients. Therefore, the UV absorbance of L,*R*- and L,*S*-BSO was measured in the separation buffer at 190 nm. Under these conditions the ratio of the absorbance coefficients for the diastereoisomers was found to be 1:1. Commercially available L-(*R*,*S*)-BSO can have a variable composition, with the ratio of the two diastereoisomers in the mixture varying from lot to lot [4]. Because only small quantities of the single isomers were available, calibration curves had to be made using measured quantities of the readily available L-(*R*,*S*)-BSO mixture. The actual stereoisomer ratio in the mixture was calculated from the measured peak areas (determined at high concentration to improve accuracy).

The assay was validated for the determination of L,*R*-BSO and L,*S*-BSO in human plasma. The high-concentration samples contained a total of 1 mg ml^{-1} BSO, and the low-concentration samples contained a total of 50 μg ml^{-1} BSO. Calibration curves for both stereoisomers were linear with $r^2 > 0.999$ ($n = 6$). Normalisation of peak area with respect to migration time was performed to reduce errors due to variation in migration velocity [24]. This procedure has a small effect on the measured R.S.D. values. In all cases, the absolute peak area R.S.D. values were found to be slightly larger than the time-normalised peak area R.S.D. values. The results of the intra- and inter-day validation are shown in Table 1. The somewhat higher R.S.D. values at the lower concentrations may be explained by the reduction in signal-to-noise ratio.

During the validation reported above, an internal standard was not used. It was not felt to be necessary since there is no extraction, protein

Table 1
Validation data

Sample (μg ml^{-1})	Intra-day ($n = 10$)		Inter-day ($n = 15$)	
	R.S.D. (%)	% of target	R.S.D. (%)	% of target
L,*R*-BSO				
High (453)	2.1	105	3.3	101
Low (22.7)	6.7	89	7.5	103
L,*S*-BSO				
High (547)	3.8	99	3.9	99
Low (27.4)	7.5	82	6.2	97

precipitation or derivatization step where errors might otherwise be introduced. Judging from the observed variability, the lack of an internal standard did not cause any major problems during the validation. In CE, the amount of sample injected is somewhat less well controlled than in HPLC with filled-loop injections, but in general it has been our experience with the equipment employed that the instrumental parameters controlling the hydrodynamic injection process (vacuum, time) introduce minimal errors in bioanalysis. However, in long-term use of this method we have found that occasionally gross injection errors do occur. Low injection seems to be sample-dependent. There is no problem with most plasma samples, but with a small minority, low injections and capillary blockage occur. It is not known why this happens, but may relate to differences in the preparation and handling of different samples. Thus in practice it has proven useful to include an internal standard to allow easy identification and rejection of analyses when a gross reduction in injection occurs. We tried a large number of possible internal standards, but most of these co-eluted with endogenous peaks. Acetaminophen was found to have suitable properties, eluting shortly after BSO in an area of the electropherogram free from interferences. A 10-μl aliquot of a 250 μg ml^{-1} aqueous solution of acetaminophen is added to 190 μl of plasma. Although acetaminophen is one of the drugs which patients are excluded from taking during the clinical trial of BSO, it is present in many non-prescription mixtures, and so the patient blank should be checked for the presence of any interfering peak.

Plasma samples were taken for pharmacokinetics measurements from a number of patients being treated with BSO and radiotherapy. All had advanced primary or metastatic carcinoma, to be treated using localized radiotherapy. All had normal hepatic and renal function. Typical electropherograms from patient samples are shown in Fig. 4. The electropherograms correspond to samples taken before infusion (blank, a) and at 1 min (b) and 60 min (c) after the end of a 10-min infusion of BSO at a dose of 1.5 g m^{-2} (the lowest dose level used in this study). The corresponding concentration–time curve for

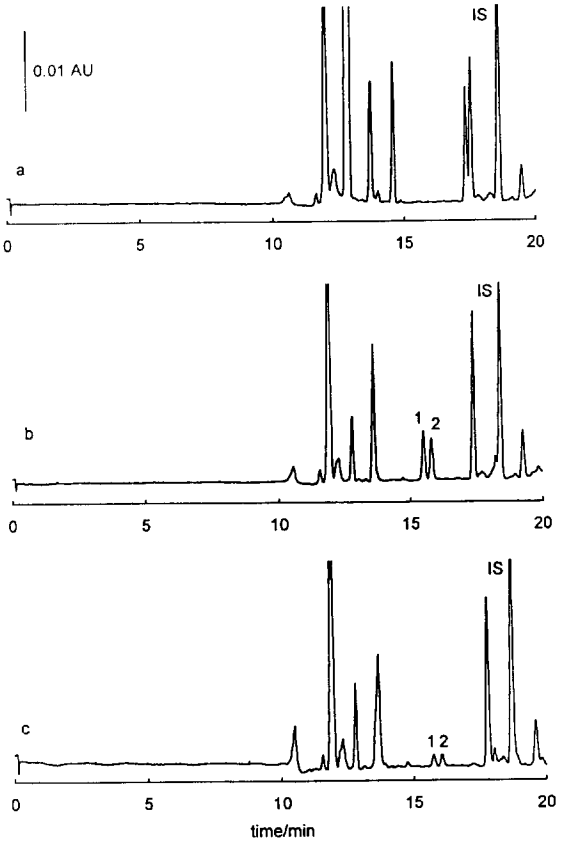

Fig. 4. Electropherograms of plasma samples from a patient receiving 1.5 g m^{-2} BSO: 1 = L,*R*-BSO; 2 = L,*S*-BSO; internal standard is large peak just to right of I.S. (a) Blank, (b) 1 min, and (c) 60 min after the end of a 10-min infusion of drug. Conditions as in text except, separation capillary, 100 cm long (78 cm to detector), 75 μm I.D.; injection, 1.5 s; field strength, 180 V cm^{-1}.

both stereoisomers is shown in Fig. 5. Maximum plasma levels were around 130 μg ml^{-1} just after the end of the infusion, and after 2 h plasma concentrations are of the order of 15 μg ml^{-1}. At the highest dose level used in this study (5 g m^{-2}) the peak plasma concentrations of BSO were as high as 590 μg ml^{-1}, dropping to around 60 μg ml^{-1} after 2 h. The initial ratio of L,*R* to L,*S* BSO is ca. 1.16:1. The ratio reduces with time so that after 1 h the plasma concentrations of both stereoisomers are approximately equal, and after 2 h there is more L,*S*-BSO than L,*R*-BSO in the plasma. This reversal was observed in all five subjects studied to date.

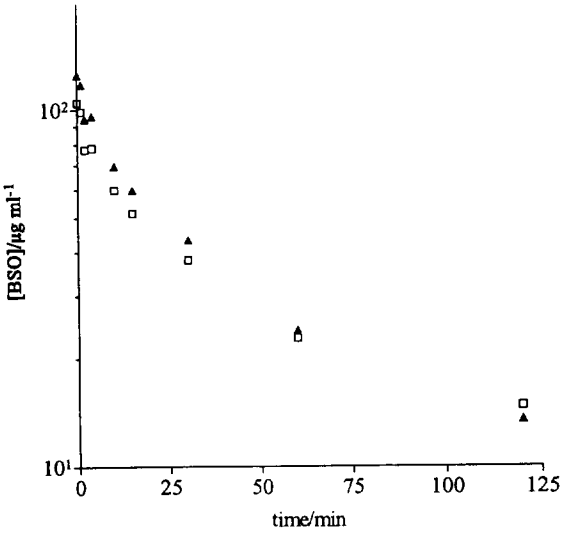

Fig. 5. Concentration of the BSO stereoisomers plotted as a function of time after the end of the infusion of the drug in a typical patient. Initially L,*R*-BSO (▲) is present at a higher concentration, but after 2 h more L,*S*-BSO (□) is present in the plasma.

4. Concluding remarks

A validated assay has been developed for the determination of clinically relevant levels of the diastereoisomers of the radiosensitizer BSO. It has advantages over previously published methods in that sample extraction and derivatization are not necessary, and that baseline resolution of the underivatized BSO diastereoisomers is achieved in approximately 15 min, with a total assay time of approximately 30 min. The sensitivity of this method is adequate for determining plasma levels of BSO in clinical trials currently being undertaken.

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