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Determination of the anticancer drug, 15-deoxyspergualin, in plasma ultrafiltrate by liquid chromatography and precolumn derivatization with naphthalene-2,3-dicarboxaldehyde/cyanide*

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Abstract: An alternative analytical method for the determination of 15-deoxyspergualin in plasma is described. The drug was initially separated from the plasma matrix by ultrafiltration and a precolumn derivatization step was performed with naphthalene-2,3-dicarboxaldehyde in the presence of sodium cyanide to yield the fluorescent *N*-substituted 1-cyanobenz[f]isoindole (CBI) derivative. The CBI derivative was separated and quantitated by reversed-phase chromatography using an ODS Hypersil column and mobile phase of KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile (48:0.8:52, v/v/v) containing dodecyl sodium sulphate (18 mM). The excitation and emission wavelengths for the fluorescence detector were 420 and 490 nm, respectively. The peak height was linearly related to drug concentration over the range from 5 ng ml^{-1} (10 nM) to 10 μg ml^{-1} (20 μM) in phosphate buffer (0.1 M, pH 7.0), spiked plasma ultrafiltrate and ultrafiltrate obtained from spiked plasma. Measurements could be made with a relative standard deviation of 4.5% or less in phosphate buffer (0.1 M, pH 7.0), 6.1% or less in spiked plasma ultrafiltrate and 12% or less in ultrafiltrate obtained from spiked plasma.

Keywords: *15-Deoxyspergualin; plasma ultrafiltrate; liquid chromatography; ion-pair; fluorescence detection; naphthalene-2,3-dicarboxaldehyde.*

Introduction

15-Deoxyspergualin (DSG), 7-[(aminoiminomethyl)amino]-*N*-[2-({4-[(3-aminopropyl)amino]butyl}amino)-(−)-1-hydroxy-2-oxoethyl] heptanamide, is a derivative of spergualin, a polyamine antibiotic, which has been isolated from the culture broths of *Bacillus laterosporus*. Spergualin has demonstrated antitumor activity against a variety of leukaemias in both *in vitro* and *in vivo* models. However, DSG has been shown to be more potent than spergualin [1]. At low doses, DSG also demonstrates antiproliferative activity against a variety of leukaemias in both *in vitro* and *in vivo* models and at high doses, DSG demonstrates immunosuppressive activity in several transplantation models [2].

Fundamental research and clinical investigations would be greatly facilitated by an efficient and practical analytical method for the determination of trace amounts of DSG in biological fluids. DSG does not display good chromophoric properties for trace analysis. The amide and guanidinium moieties demonstrate absorption maxima between 200–230 nm with molar absorptivities of approximately 10,000 M^{-1} cm^{-1} [3]. However, the analysis of DSG by direct detection lacks the specificity for trace analysis at these low wavelengths. A precolumn derivatization method using benzoin has been described by Plowman *et al.* [1]. However, this method requires extreme reaction conditions (high pH and temperature). The *gem*-carbinol amide linkage of DSG is not likely to remain intact under these conditions and therefore, the stability of the DSG derivative is questionable [4, 5]. A post-column derivatization method using *o*-phthalaldehyde has been described by Young *et al.* (to be published) and a GC–SIM method by Yamashita *et al.* [6]. Both methods allow the determination of trace amounts of DSG; however, they require involved sample preparation and specialized instrumentation such as a mass spectrometer or a post-column

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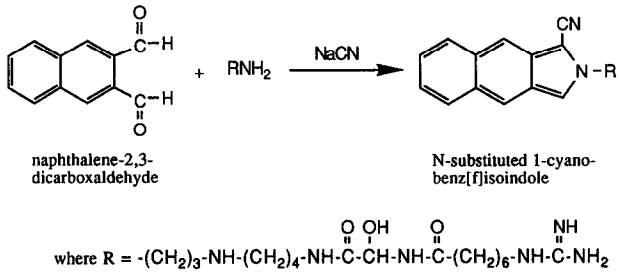


Scheme 1
The derivatization of DSG with NDA in the presence of cyanide ion.

reactor. An alternative method for the determination of DSG is described here, in which precolumn derivatization of the primary amine with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of sodium cyanide yields the fluorescent *N*-substituted 1-cyanobenz[f]isoindole (CBI) derivative (Scheme 1).

Experimental

Chemicals and reagents

All chemicals were of reagent grade and solvents of HPLC grade. A Milli-Q Water System (Millipore Corp., Bedford, MA, USA) with a 0.22-μm filter was used to deionize the water.

Samples of DSG were kindly provided by Dr Charles W. Young of Memorial Sloan-Kettering Cancer Center (New York) and Dr Jerry Phillips of the Cancer Therapy and Research Foundation of South Texas (San Antonio, TX). The DSG (100 mg DSG trihydrochloride with 100 mg mannitol) was supplied as a white lyophilized powder for injection (Nippon Kayaku Co. Ltd, Tokyo, Japan). DSG (100 mg) was reconstituted with water (3.8 ml) to make a 25 mg ml^{-1} solution from which aqueous stock solutions were made. All DSG solutions were prepared in polyethylene or polypropylene containers. Polyethylene or polypropylene pipette tips were used to transfer all DSG solutions. The DSG stock solution (25 mg ml^{-1}) was stored at −20°C when not in use.

Buffer solutions. Boric acid buffers (0.1 M) at pH values of 8.0, 9.0, 9.5 and 10.0, and phosphate buffers (0.05 M) at pH values of 11.0 and 11.5 were prepared for the DSG stability studies. For the CBI–DSG stability studies, boric acid buffers (0.1 M) at pH values of 8.0, 8.5, 9.0 and 9.5, and phosphate buffers (0.1 M) at pH values of 7.0 and 7.5 were prepared. The pH and ionic strength (0.15) of all buffer solutions were adjusted with sodium hydroxide (5 N) and sodium perchlorate (0.14 M), respectively.

Sodium cyanide solution. Solutions of sodium cyanide (10 mM) were prepared by dissolving an appropriate amount of sodium cyanide in deionized water.

Naphthalene-2,3-dicarboxaldehyde solution. Solutions of NDA (1 and 2 mM) were prepared by dissolving an appropriate amount of NDA in acetonitrile. The NDA solutions were prepared weekly and protected from light.

Stability studies

Apparatus. In the determination of DSG stability, the liquid chromatographic (LC) system consisted of a Kratos Spectroflow 400 pump (Kratos Analytical Instruments, Ramsey, NJ, USA), a Kratos Spectroflow 757 variable absorbance detector (Kratos Analytical Instruments, Ramsey, NJ, USA) and a Rheodyne injector (Rheodyne Inc., Cotati, CA, USA) fitted with a 20-μl loop. Solutions of DSG or CBI–DSG were introduced into the injection loop with a 1-ml plastic syringe. In the determination of the CBI–DSG kinetics, the LC system consisted of a Kratos Spectroflow 400 pump, a Unimetrics FS 970 LC fluorometer with a GM 970 monochromator (Kratos Analytical Instruments, Ramsey, NJ, USA) or a Shimadzu RF-530 fluorescence detector (Shimadzu Corp., Kyoto, Japan) and a Rheodyne injector fitted with a 50-μl loop. An ODS Hypersil column (5 μm, 150 × 4.6 mm) was used. Chromatograms were recorded on a Houston Instruments Omniscribe

recorder (Houston Instruments, Austin, TX, USA). A Corning Ion Analyzer 150 (Scientific Instruments, Medfield, MA, USA) was used for the pH determinations.

Chromatographic conditions. The mobile phase composition for the DSG stability study was KH_2PO_4 (0.1 M, pH 3.0)–1-hexanesulphonic acid sodium salt (5 mM)–acetonitrile (90:10, v/v) and for the CBI–DSG kinetics study, the mobile phase composition was KH_2PO_4 (0.1 M, pH 2.7)–methanol–acetonitrile (51:31:18, v/v/v). The mobile phases were filtered and degassed under vacuum. The flow rate for both methods was 1.5 ml min^{-1}. UV detection was carried out at 208 nm and the excitation and emission wavelengths for the fluorescence detector were 420 and 490 nm, respectively.

DSG stability study procedure. Aqueous solutions of DSG (0.5 mg ml^{-1}; pH 8.0–11.5) were prepared and stored at ambient temperature (22 ± 1°C) under normal fluorescent lighting. At various times aliquots (100 μl) were removed and mixed with 400 μl of phosphate buffer (0.1 M, pH 7.0). An aliquot (180 μl) of this solution was added to 4.82 ml of mobile phase and analysed by LC for DSG.

Kinetics of CBI–DSG formation and degradation. An aliquot (150 μl) of aqueous DSG (75 μg ml^{-1}) was added to 600 μl of the appropriate buffer (pH 7.5–9.5). This solution was immediately derivatized by adding 150 μl of aqueous sodium cyanide (10 mM) followed by 600 μl of NDA (1 mM). At the appropriate sampling time, an aliquot of this derivatization solution was analysed by LC for CBI–DSG. These kinetic studies were also conducted at ambient temperature (22 ± 1°C) under normal fluorescent lighting.

Plasma analysis

Apparatus. The LC system consisted of a Beckman 110B pump (Beckman Instruments, Inc., San Ramon, CA, USA), a Shimadzu RF-530 fluorescence detector and a Rheodyne injector fitted with a 50-μl loop. An ODS Hypersil column (5 μm, 150 × 4.6 mm) was used. Chromatograms were recorded on a Houston Instruments Omniscribe recorder and the data reduced with a Shimadzu C-R6A integrator (Shimadzu Corp., Kyoto, Japan).

Chromatographic conditions. The mobile phase was KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile (48:0.8:52, v/v/v) containing dodecyl sodium sulphate (18 mM). The mobile phase was filtered and degassed under vacuum. The flow rate was 2.0 ml min^{-1}. The analytical column was placed in a Shimadzu CTO-6A column oven (Shimadzu Corp., Kyoto, Japan) and maintained at 40 ± 0.1°C. The excitation and emission wavelengths for the fluorescence detector were 420 and 490 nm, respectively.

Plasma ultrafiltrate. Two methods were used for the collection of plasma ultrafiltrate. In the spiked plasma ultrafiltrate studies, large volumes of ultrafiltrate were required. Plasma was placed in the ultrafiltration device (Centriflo membrane cones, type CF25, by Amicon) and centrifuged (490*g*) for 1 h. The pH of the ultrafiltrate was adjusted to 7.0 with phosphate buffer (0.5 M, pH 6.0) prior to making calibration solutions of DSG. The ultrafiltrate was stored at −20°C when not in use.

In the spiked plasma studies, an aliquot of plasma or spiked plasma (1 ml) was placed in the ultrafiltration device [MPS-1 Micropartition System with YM30 (14 mm) membranes by Amicon] and centrifuged (800*g*) for 1 h. The ultrafiltrate was collected and stored at 4°C until analysed by LC. The ultrafiltration apparatus was washed in 0.1 N sodium hydroxide and reused. The YM30 membranes were discarded after use.

Calibration standards. Calibration solutions of DSG in phosphate buffer (0.1 M, pH 7.0), plasma ultrafiltrate and plasma were prepared at the following concentrations: 5, 10, 50, 100 and 500 ng ml^{-1} and 1, 5 and 10 μg ml^{-1}. All stock solutions and calibration standards were kept on ice (*ca* 0°C). Fresh stock solutions were prepared prior to each daily preparation of calibration standards.

Derivatization procedure. Solutions of DSG in phosphate buffer (0.1 M, pH 7.0) and plasma ultrafiltrate were derivatized by taking a 400-μl aliquot and adding 50 μl of sodium cyanide (10 mM) followed by 100 μl of NDA (2 mM). The derivatization reaction was allowed to proceed for 5 min in phosphate buffer (0.1 M, pH 7.0) and 15 min in plasma ultrafiltrate, at room temperature, at which time the reaction was quenched with 50 μl of sodium acetate buffer (0.5 M, pH 3.0). Ultra-

filtrate obtained from spiked plasma samples required the addition of a 50-μl aliquot of phosphate buffer (0.5 M, pH 6.8) to adjust the pH value to 7.0 prior to derivatization with sodium cyanide and NDA as described above.

Results and Discussion

de Montigny *et al.* developed the NDA/CN system for the precolumn derivatization of primary amines as a potential solution to the problems of product stability and variable quantum efficiencies often associated with the *o*-phthalaldehyde reagent system [7]. The NDA/CN system was originally designed for the trace analysis of amino acids and peptides, and the usefulness of the system has been demonstrated by the amino acid determination in protein hydrosylates and the determination of small peptides, such as leu-enkephalin and substance P [7, 8]. However, the original application of the NDA/CN system does not preclude its use in the trace analysis of other primary amines, such as drugs in biological fluids. Therefore, the NDA/CN system was applied in this study to the trace analysis of the novel anticancer agent, DSG.

Optimization of derivatization conditions

Initially, the present study focused on the optimization of derivatization conditions for DSG, because previous studies have shown that the rate and yield of CBI formation were maximum where the pH was equal to the pK_a of the analyte [9]. Although the pK_a values for DSG have not been determined, the pK_a of the primary amine has been estimated to be between 9–10 [10]. Consequently, the stability of DSG and its CBI derivative in alkaline media (pH 7.5–11.5) was studied to determine the optimal pH for derivatization.

The degradation of DSG was followed chromatographically with detection at 208 nm (Fig. 1) and the plots of log peak height versus time indicated that the degradation of DSG was pseudo-first order at each pH value studied. The values for the pseudo-first-order rate constants, k_{obs}, were determined in duplicate with <7.5% variation from the mean. Figure 2 shows a plot of log k_{obs} versus pH and the slope of 0.95 for this relationship, calculated by least-squares linear regression, suggested that degradation of DSG occurred by specific base catalysis over this pH range. Although the degradation products produced

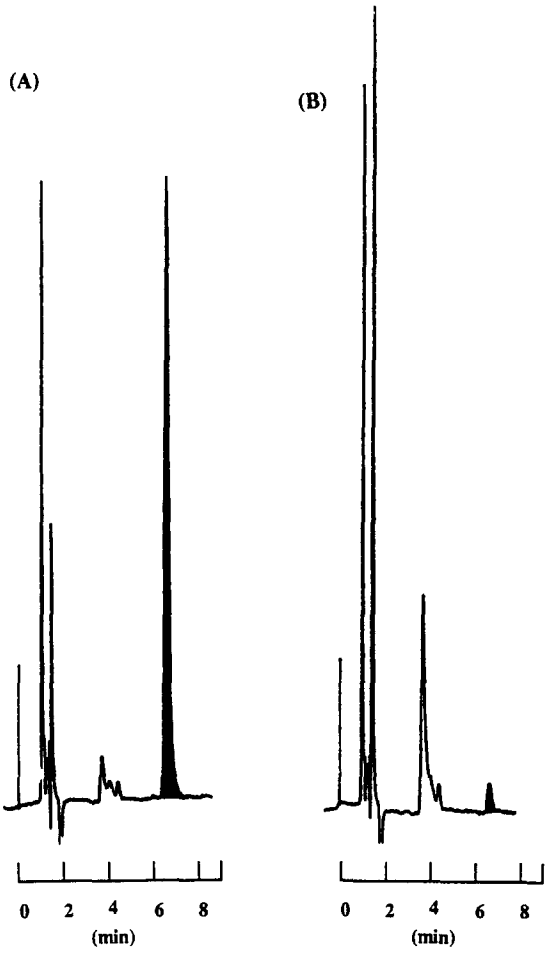


Figure 1
Representative chromatograms of DSG (0.5 mg ml^{-1}) in 0.05 M Na_2HPO_4, pH 11.5, at (A) the start of the stability experiment and (B) 4 min later.

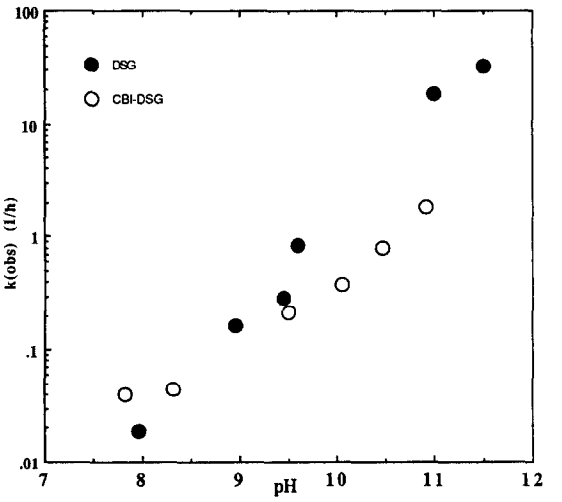
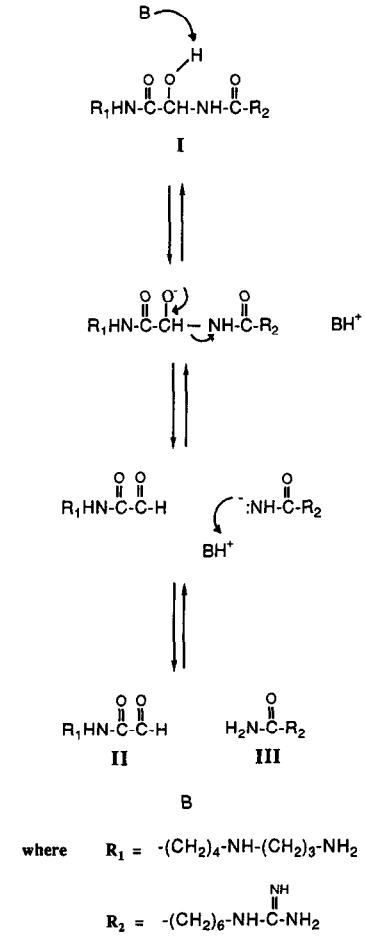


Figure 2
pH-rate profile for DSG and CBI–DSG in aqueous solution (22 ± 1°C). The DSG half-lives at pH 7.97 and 11.5 were 37.3 h and 1 min, respectively, and the CBI–DSG half-lives at pH 7.82 and 10.91 were 17.5 h and 23 min, respectively.

in alkaline media have not been isolated and identified, decomposition most likely occurs at the *gem*-carbinol amide linkage and yields 7-guanidinoheptanamide and glyoxylylspermidine [4, 5]. A proposed mechanism for the degradation of DSG is shown in Scheme 2. These data provide evidence that DSG is unlikely to survive the benzoin derivatization described by Plowman *et al.* [1]. At pH 13 the likely product of the reaction of DSG with benzoin is the fluorescent derivative of glyoxylylspermidine and not DSG. Therefore, the reaction conditions for benzoin derivatization do not appear to allow for the specificity required for DSG determination in biological fluids.

The stability of CBI–DSG was followed chromatographically with fluorescence detection at excitation and emission wavelengths of 420 and 490 nm, respectively (Fig. 3). Pseudo-first-order degradation of CBI–DSG was observed and the values for k_{obs} were obtained with a RSD of <9% (n = 3 or 4) with the exception of a 13% RSD at pH 8.32. The plot of log k_{obs} for CBI–DSG degradation versus pH was non-linear, and at each pH value the CBI derivative was more stable than the parent compound, with the exception of pH 7.82 (Fig. 2). Nevertheless, the rate of decomposition of CBI–DSG increased with increasing pH, suggesting degradation occurred by a mechanism similar to the proposed specific base catalysis of DSG itself (Scheme 2).



Scheme 2
A proposed mechanism for the degradation of DSG in alkaline media at the *gem*-carbinol amide linkage; where B is the base component, **I** is 15-deoxyspergualin. **II** is glyoxylylspermidine and **III** is 7-guanidinoheptanamide.

Above pH values of 7.0, formation of CBI–DSG was virtually instantaneous. However, formation kinetics of the derivative were observed at pH values <7.0. Figure 4 illustrates that the extent of formation of CBI–DSG as well as the stability of the derivative are dependent on the pH of the aqueous component of the derivatization reaction solution. Adequate stability of the derivative was achieved, without compromising yield, by lowering the pH to 2.5–3.0 following the initial derivatization reaction. Side products, resulting from reactions between the NDA/CN system and contaminating primary amines and condensation products formed by the NDA/CN system, are known to be fluorescent and may interfere with analytical determinations [8]. Chromatograms of derivatized phosphate buffer (0.1 M, pH 7.0), containing no DSG, demonstrated the formation of fluorescent side products and the rate of formation and the yield of these products were minimized by lowering the pH of the derivatization solution to 2.5–3.0.

The pathways which were considered when optimizing the derivatization reaction conditions for DSG are summarized in Scheme 3. A pH of 7.0 was chosen for derivatization with a reaction time of 5 min in phosphate buffer (0.1 M) and 15 min in plasma ultrafiltrate followed by a reduction in pH to 2.5–3.0.

Chromatography

The HPLC method developed to study the kinetics of DSG derivatization with NDA/CN system employed an ODS Hypersil column and a mobile phase of KH_2PO_4 (0.1 M, pH 2.7)–methanol–acetonitrile (51:31:18, v/v/v). This method provided excellent separation of CBI–DSG from its degradation products and from the components of the derivatization medium (Fig. 3). Unfortunately, this chromatographic

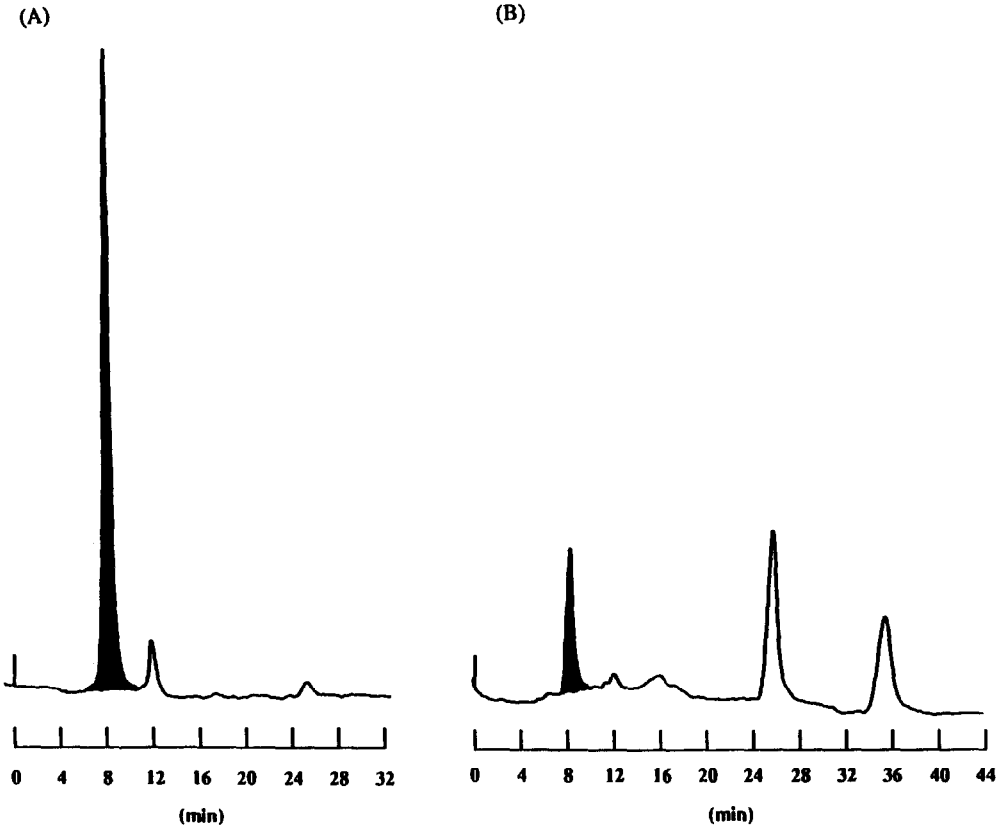


Figure 3
Representative chromatograms of derivatized DSG (18.75 μg ml^{-1}) in 0.1 M boric acid, pH 10.91, at (A) the start of the kinetics experiment and (B) 55 min later.

system did not separate CBI–DSG from the derivatized endogenous components of plasma ultrafiltrate. Therefore, an alternative approach involving ion-pair chromatography was investigated. This approach seemed particularly attractive, because CBI–DSG was considered to exist as a dication at the pH of the mobile phase. (The assignment of charge number for CBI–DSG was based on the pK_a values of analogous compounds [10].) Ion-pair chromatography has been used to provide good separation of acids, bases, ionic compounds and compounds with one or more ionizable groups [11]. Methods can be found in the literature which utilize ion-pair chromatography for the separation of naturally occurring polyamines, such as spermine, sperm-

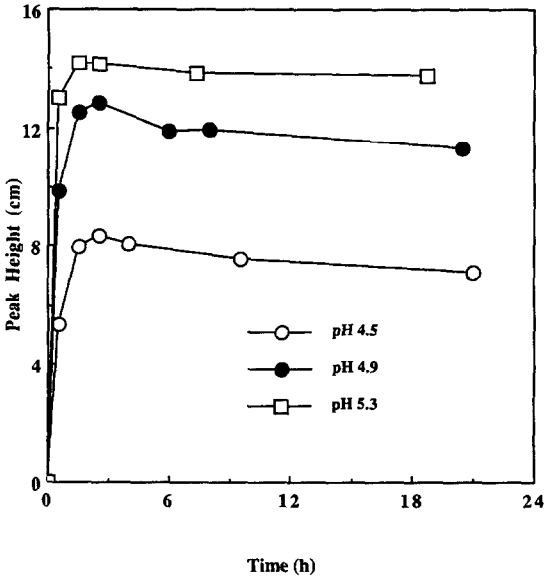
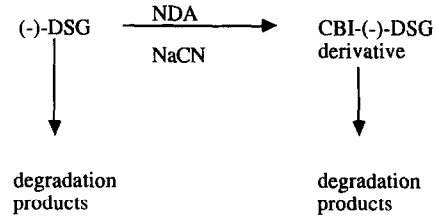


Figure 4
Formation kinetics of CBI–DSG at various pH values.



Scheme 3
Reaction pathways leading to the formation of the DSG–CBI derivative and degradation products of DSG and the DSG–CBI derivative.

idine, putrescine and cadaverine [12–15]. In particular, ion-pair chromatography has been employed for highly polar compounds, such as the polyamines described above, when separation by adsorption, reversed-phase partition or ion-exchange methods produces tailing peaks and low column efficiencies [11].

Selective increases in the retention of CBI–DSG compared with the derivatized interfering endogenous components of the ultrafiltrate matrix were achieved by adding increasing concentrations of octyl sodium sulphate (OSS) to a mobile phase of KH_2PO_4 (0.1 M), OSS (X mM)–H_3PO_4–methanol–acetonitrile (50:0.8:31:19, v/v/v/v) (Fig. 5). Baseline resolution of CBI–DSG from the plasma ultrafiltrate matrix required 7.5 mM OSS in the mobile phase. Unfortunately, the retention of CBI–DSG under these conditions was 24 min and the run time was over 50 min, because of late eluting endogenous compounds (Fig. 6).

Many theories for reversed-phase ion-pair chromatography have been developed to explain the mechanism of retention and two models are generally accepted [16–20]. In the first model, the mechanism of retention arises from ion-pair formation in the mobile phase and transfer of the neutral ion-pair into the stationary phase [19]. The second model assumes binding of the ion-pairing agent to the stationary phase, which is essentially converted to an ion exchanger [21]. The formation of a stoichiometric complex between the analyte and the ion-pairing agent is assumed by both models and the selective enhancement of retention based on analyte charge may be achieved by appropriate choices of the hydrophobicity (carbon number) and concentration of the ion-pairing agent [16]. The selective increase in the retention of CBI–DSG was attributed to the charge on the analyte (+2) being greater than the charges on the derivatized endogenous plasma ultrafiltrate components. Having been encouraged by the results of the studies with OSS, the investigation of the effects of a more hydrophobic ion-pairing reagent, dodecyl sodium sulphate (DSS), on the retention of CBI–DSG was initiated. DSS was found to be more effective than OSS in enhancing the retention of CBI–DSG. Separation of the compound of interest from components in plasma ultrafiltrate was achieved by studying the effects of the concentrations of both DSS (0.0–18 mM) and acetonitrile (45–55%) in a mobile phase containing KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile. Baseline resolution of CBI–DSG from the fluorescent endogenous components of plasma ultrafiltrate was achieved with a mobile phase of KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile (48:0.8:52, v/v/v) containing DSS (18 mM). Under these conditions, the compound of interest was the last peak to elute with a retention time of 15 min. This was considered acceptable for the routine daily analysis of more than 30 samples.

Assay validation

Standard curves for DSG in phosphate buffer (0.1 M, pH 7.0) and plasma ultrafiltrate were constructed to determine matrix effects on the extent of derivatization. Two methods were used to study these effects in plasma ultrafiltrate. In the first method, large volumes of plasma ultrafiltrate were collected, spiked with DSG, derivatized and analysed. This determined the maximum yield of CBI–DSG formed at each concentration in the ultrafiltrate matrix. In the second method, whole plasma was used to prepare calibration solutions of DSG. The ultrafiltrate was collected, derivatized and then analysed for CBI–DSG. This method determined the maximum yield of CBI–DSG formed at each concentration in the ultrafiltrate matrix after exposure to plasma proteins.

Using peak height determinations, linearity was demonstrated over the concentration range 5 ng ml^{-1} (10 nM)–10 μg ml^{-1} (20 μM) in phosphate buffer (0.1 M, pH 7.0), in spiked ultrafiltrate and ultrafiltrate obtained from spiked plasma. A RSD of 4.0% was calculated for the day-to-day reproducibility of slopes (Table 1) and a RSD of 4.5% or less (n = 3) was calculated at each concentration for a 3-day linearity study in phosphate buffer (0.1 M, pH 7.0). In spiked ultrafiltrate, a RSD of 5.4% was calculated for the day-to-day reproducibility of slopes (Table 1) and a RSD of 6.1% or less (n = 3) was calculated at each concentration for a 4-day linearity study with the exception of a 15% RSD at the 5 ng ml^{-1} level on 1 day of the study. An RSD of 12% was calculated for the day-to-day reproducibility of slopes (Table 1) and a RSD of 12% or less (n = 3) was calculated at each concentration for a 4-day linearity study in ultrafiltrate collected from spiked plasma. Representative chromatograms of derivatized phosphate buffer (0.1 M, pH 7.0) and derivatized ultra-

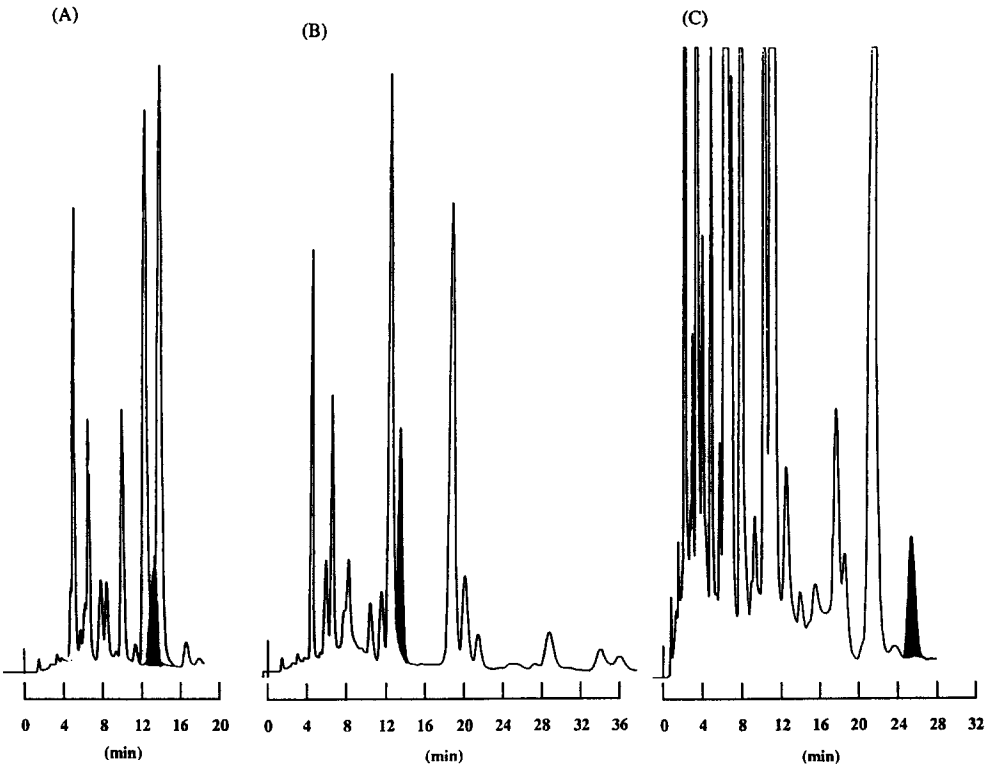


Figure 5
Representative chromatograms of derivatized components in plasma ultrafiltrate as OSS concentration changes in the mobile phase. The OSS concentrations were (A) 2 mM, (B) 5 mM and (C) 8 mM. The mobile phase composition was KH_2PO_4 (0.1 M), OSS (X mM)–H_3PO_4–methanol–acetonitrile (50:0.8:31:19, v/v/v/v).

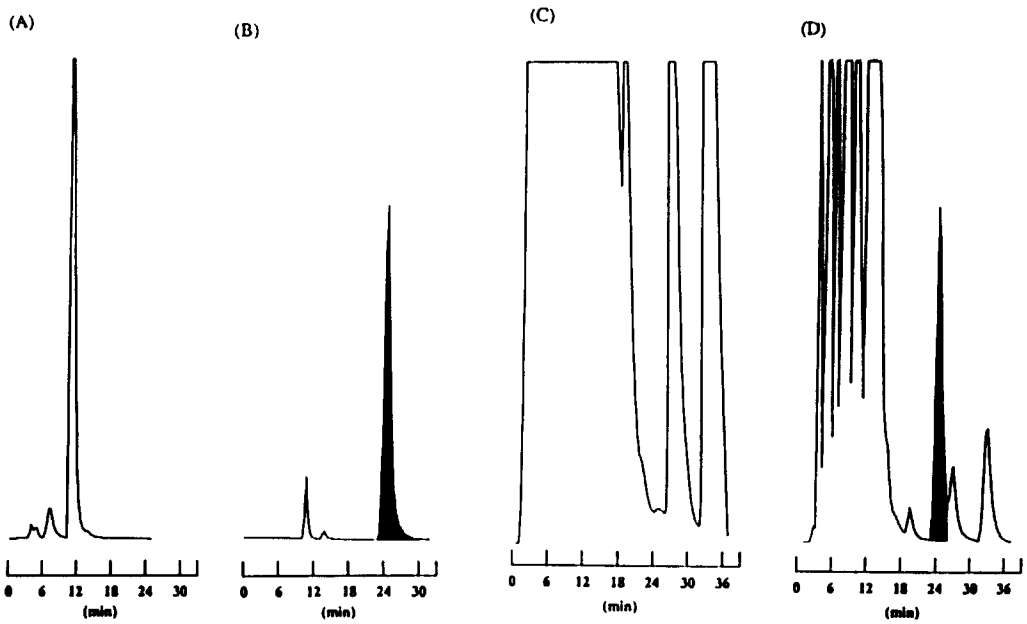


Figure 6
Representative chromatograms of derivatized phosphate buffer (0.1 M, pH 7.4) containing (A) no DSG (att. = 2^2) and (B) 10 μg ml^{-1} DSG (att. = 2^8) and derivatized ultrafiltrate obtained from spiked plasma containing (C) no DSG (att. = 2^2) and (D) 10 μg ml^{-1} DSG (att. = 2^7). The mobile phase composition was KH_2PO_4 (0.1 M), OSS (7.5 mM)–H_3PO_4–methanol–acetonitrile (50:0.8:31:19, v/v/v/v) with a flow rate of 2 ml min^{-1}. The excitation and emission wavelengths for the fluorescence detector were 420 and 490 nm, respectively.

Table 1
The day-to-day reproducibility of slopes and *y*-intercepts for the standard curves constructed in phosphate buffer (0.1 M, pH 7.0), spiked ultrafiltrate and ultrafiltrate collected from spiked plasma

	Day 1	Day 2	Day 3	Day 4	Mean	SD	RSD (%)
Phosphate buffer (0.1 M, pH 7.0)							
Slope†	23.961	23.806	22.252	NP	23.340	0.945	4.0
y-Intercept‡	616.66	99.723	104.49	NP	273.62	297.09	
R^2	0.998	1.000	1.000	NP			
Spiked ultrafiltrate							
Slope†	18.201	16.898	18.783	15.015	17.224	1.670	9.7
y-Intercept‡	−593.06	−466.32	−180.10	105.09	−283.60	311.42	*
R^2	0.999	0.999	1.000	1.000			
Spiked plasma							
Slope†	12.069	10.464	10.939	9.0289	10.624	1.259	11.9
y-Intercept‡	−36.563	−84.939	−304.22	126.20	−74.88	177.58	*
R^2	0.998	0.997	0.999	0.996			

NP = not performed.
* A Student's *t*-test was performed to determine that the intercept was not statistically different from zero at the 95% confidence level. The mean *y*-intercepts represent 0.2% of the *y*-axis.
† μV ml ng^{-1}.
‡ μV.

filtrate collected from spiked plasma are shown in Figs 7 and 8. Chromatograms of derivatized spiked ultrafiltrate were identical to those obtained for derivatized ultrafiltrate collected from spiked plasma.

The decrease in the slope of the line for the standard curve of DSG spiked into plasma, when compared with that of DSG spiked into ultrafiltrate, suggested that DSG was bound to plasma proteins. The free fraction of DSG (f_u) in plasma was calculated using two methods. The first method used the ratio of the mean slopes of the standard curve as described by equation (1):

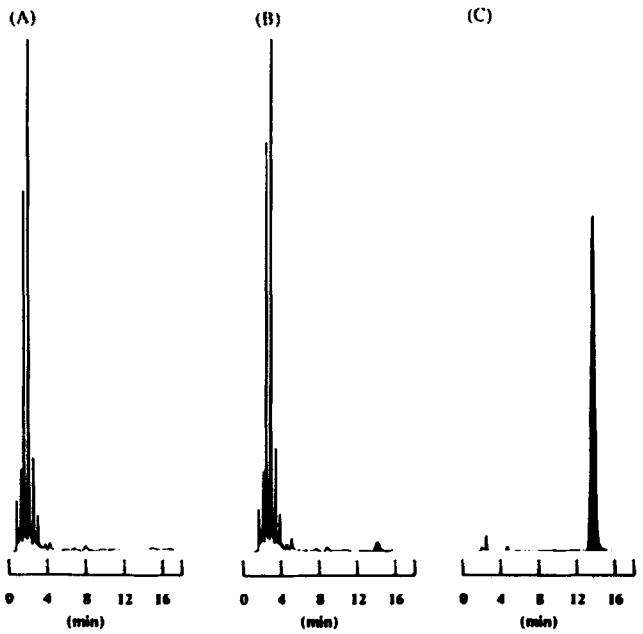


Figure 7
Representative chromatograms of derivatized phosphate buffer (0.1 M, pH 7.0) containing (A) no DSG (att. = 2^2), (B) 5 ng ml^{-1} (att. = 2^2) and (C) 10 μg ml^{-1} DSG (att. = 2^8). The mobile phase composition was KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile (48:0.8:52, v/v/v) with a flow rate of 2 ml min^{-1} and column temperature of 40°C. The excitation and emission wavelengths for the fluorescence detector were 420 and 490 nm, respectively.

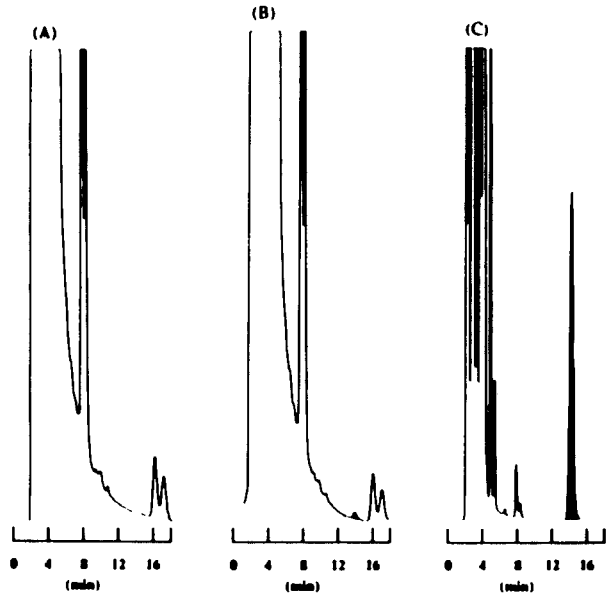


Figure 8
Representative chromatograms of derivatized ultrafiltrate collected from spiked plasma containing (A) no DSG (att. = 2^2), (B) 5 ng ml^{-1} (att. = 2^2) and (C) 10 μg ml^{-1} DSG (att. = 2^7). The mobile phase composition was KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile (48:0.8:52, v/v/v) with a flow rate of 2 ml min^{-1} and column temperature of 40°C. The excitation and emission wavelengths for the fluorescence detector were 420 and 490 nm, respectively.

Table 2
Calculation of the free fraction of DSG at each concentration of the standard curves constructed in spiked plasma and spiked plasma ultrafiltrate using mean peak height values

	Concentration (ng ml^{-1})							
	5	10	50	100	500	1000	5000	10000
Spiked ultrafiltrate								
Peak height*	71	148	768	1625	8143	17111	83516	173109
SD†	12	17	96	172	708	1406	6207	15828
RSD‡	17	12	13	11	8.7	8.2	7.4	9.1
Spiked plasma								
Peak height*	58	104	528	971	5616	9674	60506	113679
SD†	14	28	72	123	763	1197	7073	13777
RSD‡	24	27	14	13	14	12	12	12
DSG free fraction§	0.82	0.70	0.69	0.60	0.69	0.57	0.72	0.66
SD‖	0.24	0.21	0.13	0.10	0.11	0.08	0.10	0.10

*μV.
†SD (μV).
‡RSD (%) (N = 3 or 4).
§The DSG free fraction was calculated from the ratio of the peak height in spiked plasma to the peak height in spiked ultrafiltrate at a given concentration.
‖The SD was calculated from the RSD using SD = (RSD)(mean)/100. The RSD was calculated using $RSD(x/y) = [RSD(x)^2 + RSD(y)^2]^{1/2}$ [22].

$$f_u = \frac{\text{Standard curve slope for DSG spiked into plasma}}{\text{Standard curve slope for DSG spiked into plasma ultrafiltrate}} \quad (1)$$

Using this method, the free fraction of DSG was calculated to be 0.62 ± 0.10. In the second method, the free fraction of DSG was calculated using the ratio of the mean peak heights at each concentration (Table 2). This point-to-point comparison demonstrated that the calculated ratios were not statistically different from one another at each concentration nor from the ratio calculated using equation (1). Therefore, these data suggest that the protein binding of

DSG remains constant over the concentration range of 5 ng ml^{-1} to 10 μg ml^{-1} DSG.

Conclusions

A selective and sensitive method for the separation and quantitative determination of DSG in phosphate buffer (0.1 M, pH 7.0) and plasma ultrafiltrate using precolumn derivatization with NDA in the presence of cyanide ion has been described. The procedure allows for rapid sample preparation under mild conditions. Separation of CBI–DSG was achieved isocratically using an ODS column at 40°C and mobile phase of KH_2PO_4 (0.1 M)–H_3PO_4–acetonitrile (48:0.8:52, v/v/v) containing DSS (18 mM) with fluorescence detection. The application of this method to study the pharmacokinetics of DSG in New Zealand white rabbits is presently in progress.

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