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Determination of the anticancer drug metabolite WR1065 using pre-column derivatization and diode laser induced fluorescence detection

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Abstract: A liquid chromatographic (LC) procedure using alumina as stationary phase in both the pre- and the analytical column, is reported for the determination of WR1065, the active metabolite of the amino- and thiol-containing anticancer drug WR2721. After pre-column derivatization of the thiol group, the analyte is determined by LC with diode laser induced fluorescence detection in the near-infrared. Selective removal of excess label is achieved by means of column switching; it allows the detection of 5×10^{-9} M WR1065 in water and 10-fold diluted, deproteinated plasma samples. The detection limit is determined by the derivatization reaction and not by the fluorescence detection of the labelled analyte. Endogeneous thiols do not interfere.

Keywords: *WR1065; column-switching LC; diode laser induced fluorescence; pre-column derivatization.*

Introduction

The recent introduction of diode laser induced fluorescence (DIO–LIF) detection in liquid chromatography (LC) is an interesting development, especially in clinical analysis where low detection limits are required and samples are complex. Diode lasers have a number of advantages over other types of lasers (e.g. argon-ion or excimer lasers) such as a long lifetime (over 50 000 h), high stability, low cost and small size. However, commercial devices only provide laser light at wavelengths above 630 nm. These long wavelengths reduce the usefulness of diode lasers because the number of analytes that can be excited directly is limited. On the other hand, even for complex samples the background signal is low. One way to extend the applicability of DIO–LIF is chemical derivatization, which can be based on coupling of an analyte to oxazine-, thiazine- and cyanine-type fluorophores which have a high absorptivity in the near-infrared (NIR) region [1]. However, to date only a limited number of such derivatization procedures has been developed. In this study CY5.4a-IA (Fig. 1) is used, a recently developed label based on a dicarbocyanine fluorophore, with its excitation maximum at 676 nm (ϵ = 256 000 l cm^{-1} mol^{-1}) and its emission maximum at 704 nm (ϕ_f = 0.12) [2]. It contains a iodoacetamido group, which reacts selectively with thiols at pH values below 8.5.

There is a demand for selective and sensitive (bio)analytical methods to determine thiols in a wide variety of matrices [3]. This can be readily understood because thiols play an important role in biological processes such as, e.g. cellular metabolism. Moreover, sulphhydryl functions are present in various drugs. In an earlier paper, the determination of 2-mercaptobenzothiazole (MBT) after labelling with CY5.4.a-IA has been reported [2]. The main problem was the interference in the reversed-phase (RP) LC system caused by excess label. It could be solved by introducing an additional reaction with L-cysteine followed by cation-exchange LC on non-modified silica. Contrary to the derivatives formed during the labelling of MBT, the L-cysteine derivative, which contains a positively charged amino group, was strongly retained.

Following a similar approach, i.e. using cation-exchange LC, in the present study a procedure is reported which allows the selective detection of analytes containing both a

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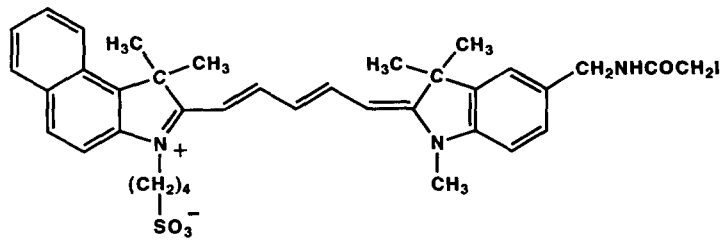

Figure 1
Structure of CY5.4a-IA.

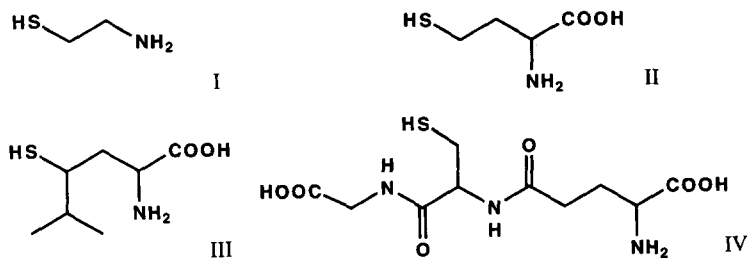

Figure 2
Structures of the amino-group containing thiols tested: (I) cysteamine; (II) L-cysteine; (III) penicillamine; (IV) reduced glutathione.

sulphhydryl and a primary amino group, such as penicillamine, cysteamine, L-cysteine and reduced glutathione (Fig. 2). Attention is focused on the detection of WR1065 (S-2-(3-aminopropylamino)ethane-thiol) (Fig. 3) [4], a compound used in both radiative and chemotherapeutic treatment of cancer. WR1065 is the active metabolite of the parent drug WR2721 (S-2-(3-aminopropylamino)ethyl-phosphorothioic acid or ethiofos) (Fig. 3). WR1065 and the corresponding disulphide (WR33278) are formed endogeneously by a fast dephosphorylation reaction of the parent drug. WR2721 was developed by the Walter Reed Army Hospital (Washington, DC, USA) to protect soldiers against radioactive radiation in case of a nuclear attack. It inhibits the formation of oxygen radicals and repairs the damage of radicals on DNA by means of a proton-donating reaction [5]. Its usefulness in cancer treatment is mainly based on the selective protection of non-tumour tissue against radioactive radiation. This selectivity is based on the fact that in non-tumour tissue the conversion of WR2721 into WR1065 is rapid and results in accumulation of the active metabolite. In tumour tissue conversion is much slower as a result of the diminished activity of alkaline phosphatase [6]. WR2721 is also used as a modulator of the cytostatic

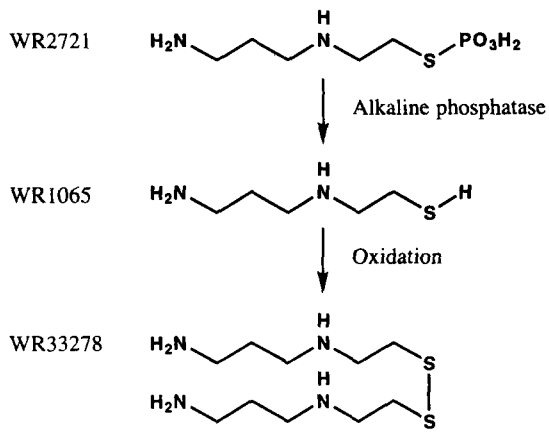

Figure 3
Metabolic conversion of WR2721 into WR1065 and WR33278.

agents carboplatin and cisplatin; it effectively diminishes the toxicity of the latter compound in several tissues without affecting the antitumour activity [7]. The therapeutic concentration of WR2721 is very high (150 mg kg^{-1}), and results in plasma concentrations of WR1065 in the order of (1–5) $\times$ 10^{-5} M immediately after administration. Clinically interesting concentrations of WR1065 are $\geq 1 \times 10^{-7}$ M [8]. However, WR2721 has a half-life of less than 5 min in plasma, which is short compared to that reported for WR1065 (about

45 min) [6]. This difference is due to the release of WR1065 from a mixture of disulphides that is formed shortly after administration [9]. Since WR1065 is assumed to be the only active metabolite, its selective detection is very important for the dosimetry of either radiation or chemotherapeutic compounds. In the literature, WR1065 is determined by RPLC with amperometric [10] or fluorescence detection after derivatization with monobromobimane [4].

However, using the above detection techniques sensitivity and/or selectivity in real samples were not fully satisfactory. In this paper an alternative detection method is described, which allows the sensitive detection of thiols containing amino groups with emphasis on WR1065.

Experimental

Equipment

Thin-layer chromatography (TLC) was performed using either Silica 60, with 0.25 mm particles (Merck, Darmstadt, Germany), or Alumina 60 with 0.25 mm particles (Merck) as the sorbent.

The column-switching LC set-up is schematically depicted in Fig. 4. It consisted of a Model 951 Tracor (Austin, TX, USA) high-pressure pump (for Eluent I), a home-made six-port injection valve (Free University, Amsterdam, the Netherlands) equipped with a 25-μl loop (loop 1), a home-made six-port switching valve equipped with a stainless-steel precolumn (10 × 3.1 mm i.d.) slurry packed (methanol) with 20 μm Alox T20 (Merck) (loop 2). Loop 1 was used for sample introduction, while loop 2 was used to switch from the loading (Eluent I) to the desorption and analytical separation (Eluent II) position. For Eluent II, a Model 300 Gynkotek (Germering-München, Germany) high-pressure pump was used in combination with a 100 × 3.1 mm i.d. slurry packed (methanol) 10 μm Alox T10 analytical column (Merck). The precolumn was used at room temperature, while the analytical column was thermostatted at 50°C. Further details of the column-switching procedure and the composition of Eluents I and II will be discussed below.

The DIO-LIF detection system was similar to that described in [2]. Excitation was performed with a LAS 200-670-10 diode laser (Lasermax, Rochester, NJ, USA), which provides 9.5 mW at 670 nm. The fluorescence light was collected via a 1000 μm (600 μm core diameter) optical fibre (Quartz & Silice, Uithoorn, the Netherlands). One end of the fibre was inserted in the flow cell and tightly locked into position by a home-made finger-tight at a distance of 2 mm from the laser beam. The fluorescence collected by the fibre was focused with a 2.5-cm lens (f/# = 1) on a Model C-31034 GaAs photomultiplier (PMT) (RCA, Lancaster, UK) after passing a 3 mm Schott RG695 cut-off filter. The PMT was controlled by a Model 456H high-power supply (EG&G Ortec, Danvers, MA, USA) and used at 1750 V. Cooling was provided by a home-made temperature controller unit set at −20°C. Data registration was performed with a Model C-10 photoncounter (Thorn EMI, Fairfield, NJ, USA) and a BD-40 recorder (Kipp & Zoonen, Deventer, the Netherlands).

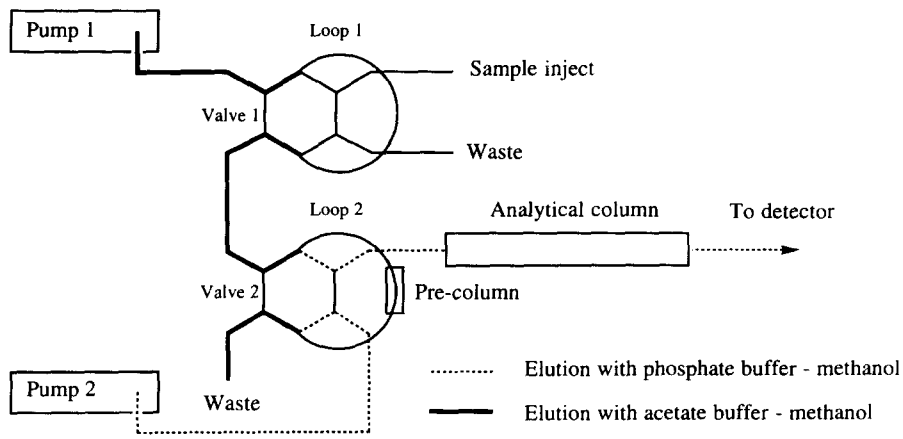

Figure 4
Schematic representation of the column-switching LC system.

Reagents

HPLC-grade methanol and high-purity acetic acid were purchased from J.T. Baker (Deventer, the Netherlands) and were freshly distilled before use. Demineralized water (Milli-Q Purification System, Millipore, Bedford, MA, USA) was used throughout this study. CY5.4a-IA was synthesized according to the procedure described in [2]. WR1065 was a gift from the Academic Hospital of the Free University. All other reagents used were of the best quality available. The inorganic compounds were supplied by J.T. Baker, while the organic solutes were purchased from Janssen Chimica (Beerse, Belgium). Plasma and urine samples were obtained from healthy volunteers and were supplied by the Academic Hospital of the Free University.

Methods

Stock solutions of 10^{-3} M WR1065 were prepared in a 0.01 M potassium dihydrogen phosphate buffer (pH 3.0) containing 5 mM EDTA and stored at −20°C. Under these conditions the solution was stable for at least 3 months (less than 5% loss). Stock solutions (10^{-3} M) of the other thiols were prepared in the same solvent and stored at −5°C for maximally 2 weeks to avoid dimer formation [4]. The stock solutions of the thiols were diluted to the appropriate concentration with a 5 mM phosphate buffer (pH 3.0) containing 5 mM EDTA within 1 day before derivatization. A stock solution (2×10^{-4} M) of CY5.4a-IA was made in 0.20 M ammonium carbonate buffer (pH 8.0)–methanol (25:75, v/v) and stored in the dark at room temperature. This solution was stable for more than one week.

Derivatization of the thiols was performed by mixing 500 μl of the diluted stock solution of the analyte with 500 μl of the reagent in a 2-ml gas-tight glass reaction vial. After bubbling with dry nitrogen for 1 min, the mixture was heated for 1 h at 65°C. After cooling to ambient temperature, the mixture was diluted with the eluent to a concentration of 10^{-8}–10^{-10} M and 25 μl were injected onto the LC column.

Urine samples were filtered over a 0.2 μm FP030/30 filter (Schleicher & Schuell, Dassel, Germany) before derivatization. Pooled human plasma samples were first deproteinated by 15 min centrifugation with four volumes of methanol at 2500 rpm. Next, a 500 μl aliquot of the supernatant was spiked with WR1065, mixed with 250 μl of 0.20 M ammonium carbonate buffer (pH 8.0) and 250 μl of the label solution. Further treatment was performed as described above.

Results and Discussion

Separation

The main problem that had to be solved in the present study was the separation of the excess CY5.4a-IA label from the derivatized analytes. Before developing an appropriate column-switching LC system, TLC experiments were performed to acquire some insight into the chromatographic problems that had to be expected.

TLC Experiments. Bare silica can be used at pH 5.5, i.e. in the cation-exchange mode, to separate labelled thiols (containing an amino group) from the unreacted label. Since primary and secondary amino groups usually have a positive charge at pH <7 (Table 1), labelled analytes containing such a functionality are retained much stronger than the non-charged CY5.4a-IA label. To determine their retention characteristics, for a number of analytes TLC experiments were performed on both silica and alumina using mixtures of methanol and either a 0.50 M sodium acetate buffer or a 0.10 M potassium phosphate buffer at various pH values as eluents.

Stock solutions (10^{-3} M) of cysteamine, L-cysteine, reduced glutathione, penicillamine and WR1065 were derivatized with a 10^{-3} M solution of CY5.4a-IA. The derivatization reaction was checked for completeness by TLC on silica or alumina. When using 50 vol.% of methanol or more in the eluent, the R_f value of the unreacted label was larger than 0.70, for all conditions tested (Tables 2–4). At lower methanol percentages the label mainly determined the retention behaviour of the deriv-

Table 1
pK_a values (in water) of the amino group-containing thiols studied [11]

	pK_1	pK_2	pK_3	pK_4
Reduced glutathione	9.52	8.65	3.61	2.12
L-cysteine	10.11	8.13	1.90	—
Penicillamine	10.46	7.97	2.44	—
Cysteamine	10.79	—	—	—
WR1065	10.14	7.26	—	—

Table 2
R_f values of the thiol derivatives on silica, using 0.50 M acetate buffer (pH 5.5)–methanol as eluent

Analyte	R_f value for methanol				
	50%	60%	70%	80%	90%
Reduced glutathione	0.70	0.70	0.70	0.70	0.80
L-cysteine	0.70	0.55	0.55	0.50	0.60
Penicillamine	0.70	0.70	0.65	0.65	0.50
Cysteamine	0.40	0.50	0.50	0.45	0.20
WR1065	<0.03	<0.03	<0.03	<0.03	<0.03
CY5.4a-IA	0.70	0.70	0.70	0.70	0.80

Table 3
R_f values of the thiol derivatives on alumina, using 0.50 M acetate buffer (pH 5.5)–methanol

Analyte	R_f value for methanol				
	50%	60%	70%	80%	90%
Reduced glutathione	<0.03	<0.03	<0.03	<0.03	<0.03
L-cysteine	0.05	<0.03	<0.03	<0.03	<0.03
Penicillamine	0.15	0.15	0.05	<0.03	<0.03
Cysteamine	0.65	0.55	0.55	0.45	0.15
WR1065	0.70	0.45	<0.03	<0.03	<0.03
CY5.4a-IA	0.95	0.90	0.90	0.90	0.90

Table 4
R_f values of the thiol derivatives on alumina, using 0.10 M phosphate buffer (pH 5.5)–methanol

Analyte	R_f value for methanol				
	50%	60%	70%	80%	90%
Reduced glutathione	0.05	<0.03	<0.03	<0.03	<0.03
L-cysteine	0.10	0.05	<0.03	<0.03	<0.03
Penicillamine	0.20	0.10	<0.03	<0.03	<0.03
Cysteamine	0.45	0.45	0.45	0.30	0.05
WR1065	<0.03	<0.03	<0.03	<0.03	<0.03
CY5.4a-IA	0.95	0.90	0.90	0.90	0.90

atives and separation from excess label was difficult.

On silica, an increase of the methanol content from 50 to 90 vol.% at pH 5.5 (Table 2) caused a decrease in R_f for cysteamine, L-cysteine and penicillamine derivatives. The nature of the buffer ions, acetate or phosphate, had no influence. The R_f of the reduced glutathione derivative hardly changed, while the WR1065 derivative remained at the origin in all instances. Obviously, under the conditions used, silica acted as a weak cation exchanger. The WR1065 derivative was strongly retained because of its double positive charge at pH 5.5. The monovalent cysteamine derivative had the weakest retention. Since reduced glutathione, L-cysteine and penicillamine contain at least one carboxylic acid function in addition to the amino group, the retention behaviour of their derivatives is not easily predicted. In addition to steric effects, the type and concentration of the buffer ions may also play a role.

On alumina the retention behaviour was completely different (Tables 3, 4). This may be due to the fact that the buffer pH (for both acetate and phosphate) was below the isoelectric point of alumina, which means that the sorbent carried a positive charge under the conditions used. This suggestion is substantiated by the fact that, whereas the label itself showed almost no retention, all the derivatives were retained to a certain extent. From among the derivatives, the cysteamine derivative showed the least retention, while the derivatives of the three analytes containing both an

amino and a carboxylic acid function were strongly retained. Except for the WR1065 derivative, the retention behaviour was essentially the same whether using a acetate or a phosphate buffer.

With a phosphate buffer, the WR1065 derivative was strongly retained if 50 vol.% methanol was present in the eluent; with an acetate buffer, the compound had a high R_f value. This is rather unexpected because in both buffer solutions the analyte as well as the stationary phase should be positively charged, which implies that the retention should be low. Obviously, phosphate ions play a specific role. Possibly, they form complexes with the WR1065 derivative and cause a partial neutralization of the positive charge.

Column-switching LC. For the analytes tested, the above TLC experiments indicate that when amino group-containing thiols are studied, the excess label can be removed after the labelling reaction by LC on bare alumina, utilizing a change in the percentage modifier. After removal of the unreacted label with 0.50 M acetate buffer–methanol (10:90, v/v), desorption can be performed with for instance 0.50 M acetate buffer–methanol (50:50, v/v). Elution over an alumina analytical column with the same eluent enables a separation of the various retained thiol derivatives. However, the data of Table 4 suggest an interesting alternative for WR1065: the combination of an alumina precolumn and an alumina analytical column, which allows the selective determination of derivatized WR1065 simply by changing the *nature* of the buffer solution, without a change in modifier concentration or pH. The WR1065 derivative will be strongly retained on the precolumn when using a phosphate buffer (Eluent I), while the excess label has no notable retention under these conditions and can be flushed to waste. Next, the WR1065 derivative (and derivatives of other amino group-containing thiols present in the sample) are transferred to the analytical column in the backflush mode, using an acetate buffer (Eluent II) (Fig. 4). For both eluents pH, buffer concentration, and modifier percentage were optimized.

Eluent I. With 0.05 M phosphate buffer (pH 5.5)–methanol as the eluent, removal of the excess label required less eluent if the modifier concentration was kept low (50 vol.%); as a result switching to the analytical column could be performed faster. However, next to the WR1065 derivative, derivatives of endogeneous thiols present in plasma were also retained. Fortunately, these potentially interfering compounds were easily separated from the WR1065 derivative on the analytical column. Methanol percentages of less than 50 vol.% (v/v) were not used for reasons outlined above.

As regards the phosphate buffer concentration, 0.01 M instead of the earlier 0.05 M appeared to be sufficient for nearly quantitative removal of the excess label. For both phosphate buffer concentrations, less than 0.1% of the total amount of injected label was left on the precolumn after eluting for 3 min with phosphate buffer (pH 5.5)–methanol (50:50, v/v) (flow rate, 1.0 ml min^{-1}) as was determined after subsequent elution with the optimized Eluent II (see below). Changing the pH of the eluent had hardly any effect in the pH 3–7 range. Therefore, during all further experiments the composition of Eluent I was 0.01 M phosphate buffer (pH 5.5)–methanol (50:50, v/v).

Eluent II. For Eluent II which will be used for both desorption from the precolumn and separation on the analytical column, the influence of the percentage modifier on the retention of the WR1065 derivative and the derivatives of the endogeneous thiols was studied. With 0.25 M acetate buffer (pH 5.5)–methanol as eluent, desorption of the precolumn was complete within 1 min (flow rate, 0.75 ml min^{-1}) for methanol percentages below 90 vol.%. The retention of the WR1065 derivative on the analytical column was found to increase when increasing the methanol percentage. This may well be due to the corresponding decrease of the real buffer concentration, which causes increased retention when cation-exchange chromatography is used. Increasing the buffer concentration from 0.01 to 0.50 M resulted in an almost linear decrease of the retention of the WR1065 derivative. Higher buffer concentrations did not effect a further decrease of retention. The influence of the acetate buffer on the retention of the WR1065 derivative is strongly related to the concentration of free acetate ions in the eluent. An increase in the pH of the eluent by the addition of a strong base resulted in shorter retention times. However, when the pH was

changed while adjusting the buffer concentration to keep the amount of free acetate ions constant, no pH influence was observed. In view of these results, the final composition of Eluent II was 0.25 M acetate buffer (pH 5.5)–methanol (20:80, v/v). With this eluent, the interfering compounds were either eluted close to the unretained peak or not at all within the time of analysis (cf. below).

To summarize, column-switching LC was performed with 0.01 M phosphate buffer (pH 5.5)–methanol (50:50, v/v) during preconcentration, at a flow rate of 1.0 ml min^{-1}. During backflush desorption and the actual analysis, elution was performed with 0.25 M acetate buffer (pH 5.5)–methanol (20:80, v/v) at a flow rate of 0.75 ml min^{-1}.

Derivatization

The effect of pH and temperature on the derivatization of WR1065 with CY5.4a-IA were studied using the reaction conditions outlined in the *Experimental* section. Figure 5 shows the pH influence at a reaction temperature of 70°C; Fig. 6 shows the influence of the reaction temperature at pH 8.0. As expected the reaction is faster both at higher temperatures and higher pH values. Temperatures higher than 70°C were not used to avoid boiling of the reaction mixture. At higher pH a higher degree of deprotonation of the thiol is effected and the rate of nucleophilic substitution increases, which results in a higher derivatization yield because side-reactions like the formation of disulphides are less favoured by the pH increase (Fig. 5). The main drawback of performing the reaction at pH values over 8.0 is the loss of selectivity, because of the simultaneous labelling of thiols and primary amines. Therefore, pH 8.0 was used in all further studies. Under optimum conditions, 70°C and pH 8.0, the reaction proceeded quantitatively within 20 min. The derivatization yield was linear for analyte concentrations between 5×10^{-9} M and 1×10^{-5} M; the calibration curve, containing 11 data points measured in duplicate, is given by: $y = 1.21(\pm 0.03)x + 10.1(\pm 2.3)$ ($r^2 = 0.996$), where x is the analyte concentration multiplied by 10^8 and y the relative amount of fluorescence from the analyte as observed with DIO-LIF detection. Below 5×10^{-9} M, the derivatization yield decreased rapidly and quantitative conversion could not be effected anymore even if the reaction time (to 60 min) or the excess reagent

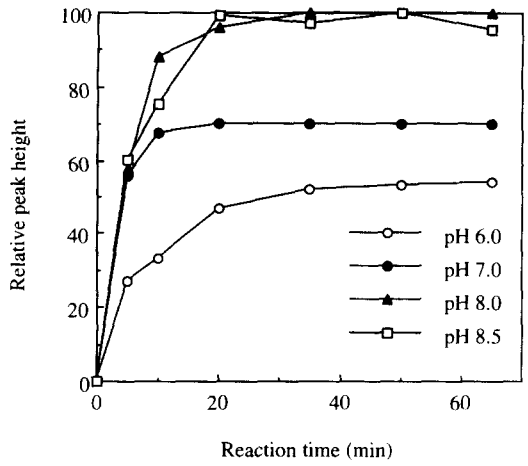

Figure 5
Influence of pH on the reaction yield of the derivatization of WR1065 with CY5.4a-IA at 70°C; see text for further details.

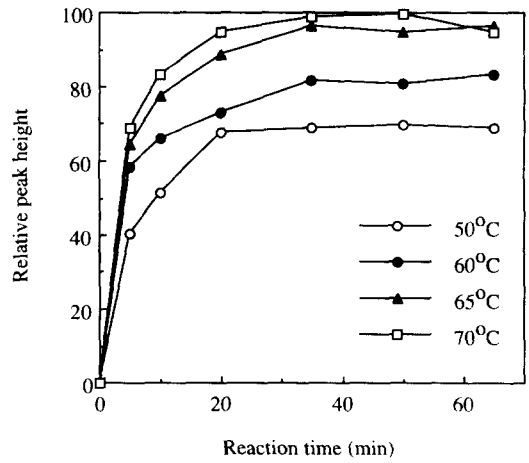

Figure 6
Influence of temperature on the reaction yield of the derivatization of WR1065 with CY5.4a-IA at pH 8.0; see text for further details.

(to 5×10^{-4} M) was increased: in other words, it is the derivatization reaction which determines the detection limit (injected concentration) of WR1065. The DIO–LIF detection system is not the limiting factor. Actually, if a 10^{-5}–10^{-7} M solution of WR1065 was derivatized with CY5.4a-IA and, next, diluted, the detection limit was $\sim 1 \times 10^{-12}$ M or about 10^3-fold better than under real trace-level conditions.

Plasma samples

It is known that due to the presence of certain enzymes, WR1065 is not stable in plasma samples [12]. In order to study this

aspect, plasma was spiked with 1×10^{-5} M and 5×10^{-5} M of WR1065 and then deproteinated (which resulted in 10-fold dilution; cf. *Methods* section) after various time intervals. Subsequently, derivatization was performed. The data of Fig. 7 show that the analyte concentration decreases very rapidly. Evidently, plasma samples must be deproteinated immediately after collection.

When deproteinated plasma is analysed (as was the procedure in all further experiments), as a result of the sample pretreatment, WR1065 was actually spiked in 10-fold diluted deproteinated plasma and then derivatized with CY5.4a-IA under the optimum conditions described above. LC chromatograms obtained after the derivatization of such plasma, spiked with 1×10^{-8} M and 2×10^{-8} M WR1065, respectively, are shown in Fig. 8. The samples were 100-fold diluted with the LC-eluent before injection into the LC — DIO–LIF detection system to avoid overloading of the photomultiplier. No undue interferences from derivatized endogeneous thiols were observed. Only a very small, and unidentified, peak showed up in the blank sample, which eluted slightly in front of the WR1065 derivative, which is not observed if spiked water samples were analysed. In order to prevent problems with precolumn fouling and/or memory effects, the alumina precolumn was exchanged after ten analyses. The fact that no interfering thiol (e.g. L-cysteine, reduced glutathione) derivatives are observed in the chromatogram can be explained on the basis of the TLC results presented above. These show that when an acetate buffer is used, the retention of the WR1065 derivative is significantly less than that of the derivatives of the other thiols studied.

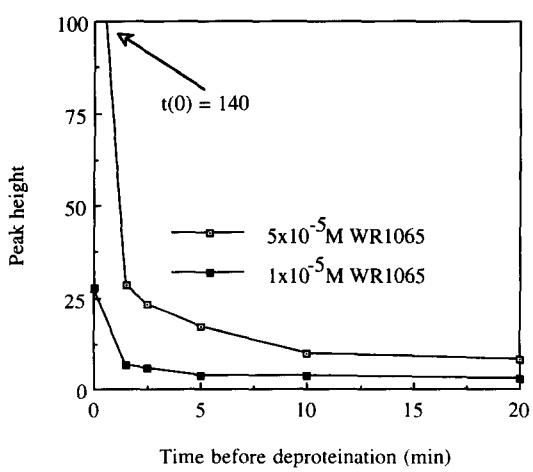

Figure 7
Decompostion of WR1065 in plasma (spiking levels: 1×10^{-5} M and 5×10^{-5} M). After spiking, the samples were left standing at room temperature for the period of time indicated and then deproteinated and subsequently derivatized.

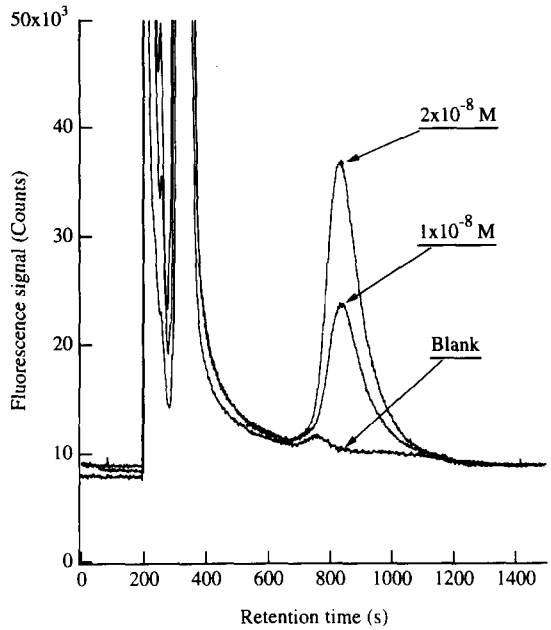

Figure 8
LC — DIO–LIF chromatograms of (10-fold diluted) deproteinated plasma samples after derivatization with CY5.4a-IA. Results are shown for both blank plasma and for plasma samples spiked with 1×10^{-8} M WR1065 and 2×10^{-8} M WR1065.

Analysis of 10 plasma samples, spiked at the 5.0×10^{-7} M level, gave an RSD of 7.6%. Linearity of response was observed for analyte concentrations between 5×10^{-9} M and 1×10^{-5} M. The calibration curve, containing eight data points measured in duplicate, is given by: $y = 1.17(\pm 0.04)x + 6.1(\pm 3.3)$ ($r^2 = 0.994$), where x is the analyte concentration multiplied by 10^8 and y the relative amount of fluorescence from the analyte as observed with DIO–LIF detection. The detection limit in 10-fold diluted plasma samples is 5×10^{-9} M or 0.1 pmol injected. In other words, the method developed is sensitive enough to fulfil clinical requirements ($\geq 1 \times 10^{-7}$ M) [7].

Conclusions

A selective and sensitive method has been developed for the determination of WR1065 in biological samples. After pre-column labelling

with CY5.4a-IA, column-switching LC in combination with near-infrared DIO–LIF detection is used for the selective determination of the WR1065 derivative. The WR1065 derivative is selectively retained on an alumina precolumn when elution is performed with 0.01 M phosphate buffer (pH 5.5)–methanol (50:50, v/v) (1.0 ml min^{-1}), while 99.9% of the excess label is flushed to waste within 3 min. Desorption is achieved by elution with 0.25 M acetate buffer (pH 5.5)–methanol (20:80, v/v) (0.75 ml min^{-1}); separation on the alumina analytical column takes ca 20 min. With this procedure, WR1065 can be quantified at concentrations as low as 5×10^{-9} M. This lower limit of detection is a result of the kinetics of the labelling reaction (cf. [2]) rather than a result of the sensitivity of the present DIO–LIF detection system. Actually, detection limits for samples labelled at high analyte concentrations and, next, diluted are 1×10^{-12} M.

Owing to the low stability of WR1065 in plasma, plasma samples had to be deproteinated before labelling, which caused a 10-fold dilution. However, even so, detection limits were 10-fold lower than required for clinical studies and no interfering peaks showed up in the chromatograms.

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