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High-performance liquid chromatographic assay using electrochemical detection for the combined measurement of amifostine, WR 1065 and the disulfides in plasma

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the combined analysis of the chemoprotective agent, amifostine, its active metabolite, WR 1065, and the (symmetrical and mixed) disulfides of WR 1065 in plasma. These three compounds were quantified by measuring WR 1065 after three different sample pretreatment procedures. During these procedures, amifostine and the disulfides were quantitatively converted into WR 1065, by incubating the sample either at a low pH or in the presence of dithiothreitol, respectively. The resulting amounts of WR 1065 were determined by HPLC with electrochemical detection (Au electrode, +1.00 V). The lower limit of quantitation of WR 1065 was 0.15 μM. The within-day and between-day precision were ≤4.4 and ≤8.2% for WR 1065, ≤4.9 and ≤13.1% for amifostine and ≤8.5 and ≤5.5% for the disulfides, respectively. The within-day and between-day accuracy ranged from 97.2 to 109.8% and from 97.6 to 101.5% for WR 1065, from 88.3 to 110.7% and from 99.4 to 101.5% for amifostine and from 99.2 to 110.2% and from 103.3 to 104.9% for the disulfides, respectively. This method is superior to other described methods due to its simple and relatively rapid analysis of all three compounds in one system. Furthermore, it is at least as sensitive as earlier reported methods for one of the compounds and the application of the gold electrode requires only minor maintenance. Therefore, this method is very suitable for pharmacokinetic studies of amifostine and its metabolites. As an example, the plasma concentrations of amifostine, WR 1065 and the disulfides are shown in a patient after receiving an i.v. dose of 740 mg/m^2 amifostine.

Keywords: Amifostine; WR 1065

1. Introduction

In clinical and preclinical studies, amifostine [S-2-(3-aminopropylamino)-ethylphosphorothioic acid, WR 2721, Ethyol], originally developed as a radioprotector, was shown to protect against chemotherapy-induced toxicities, without reducing anti-tumor activity [1–3]. This selective protection was thought to be based on the preferential formation and uptake of the active metabolite, WR 1065, in normal tissues (Fig. 1) [4]. The formation of this dephosphorylated free sulfhydryl metabolite is catalyzed by alkaline phosphatase. Subsequently, WR 1065 can be oxidized to its symmetrical disulfide, WR 33278, or to mixed disulfides with endogenous thiols like glutathione and cysteine or thiol-containing proteins (Fig. 1). Due to the reversibility of the disulfide bond, the

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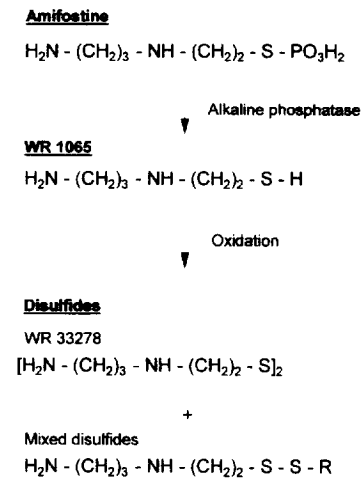


Fig. 1. Structural formula of amifostine and its conversion into the main metabolites, WR 1065 and disulfides (with WR 1065 itself or endogenous thiols (RSH))

(mixed) disulfides may be regarded as an exchangeable pool of WR 1065.

To study the pharmacokinetics and metabolism of amifostine, an accurate and sensitive assay for amifostine, WR 1065 as well as the disulfides in plasma is required. High-performance liquid chromatography (HPLC) methods for the analysis of one or two of these compounds have been described, most of them are based on fluorimetric [5–8] or electrochemical detection [9–14]. The drawback of most of the described procedures is the low sensitivity and the absence of a simple procedure for the combined analysis of all three compounds. A combined analysis of amifostine and WR 1065 was difficult due to the quite different chromatographic behaviours of the two compounds [10]. Therefore, in some assays, amifostine or the disulfides were converted into WR 1065 before analyzing the WR 1065 concentration, in order to measure both compounds with a single HPLC procedure. The disulfides could be reduced to WR 1065 by addition of tri-*n*-butylphosphine [13] or dithiothreitol [7] or electrochemically (only the symmetrical disulfide WR 33278) [14], whereas conversion of amifostine to WR 1065 was performed with alkaline phosphatase [7]. However, until now, none of the described methods succeeded in combining these options into one sensitive, simple, accurate and fast analytical method, suitable for studying the pharmacokinetics of amifostine and its metabolites with one system.

Therefore, the aim of this study was to develop a simple, accurate and sensitive method for the combined analysis of amifostine, WR 1065 and the disulfides.

The conversion of amifostine into WR 1065 was performed by incubation under acidic conditions, whereas the reduction of the disulfides occurred in the presence of dithiothreitol. A sensitive electrochemical detector provided with a wall-jet Au electrode was used for the detection of WR 1065. This paper describes the procedure and the validation of the assay. It also shows the concentrations of all three compounds obtained in the plasma of a patient treated i.v. with a dose of 740 mg/m^2 amifostine.

2. Experimental

2.1. Materials

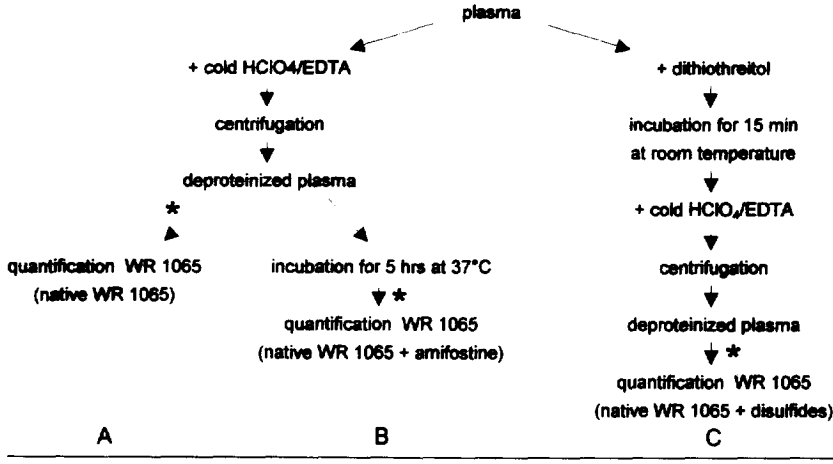
Amifostine [S-2-(3-aminopropylamino)phosphorothioate, trihydrate], WR 1065 [2-(3-aminopropylamino)ethanethiol, dihydrochloride] and WR 1729 [N-(2-mercaptoethyl)-1,5-pentanediamine dihydrochloride] were provided by US Bioscience (West Conshohocken, PA, USA). Monochloroacetic acid (ClAcH) was obtained from Fluka Chemie (Bornem, Belgium), 1-hexanesulfonic acid, sodium salt was from Acros (Geel, Belgium), EDTA disodium salt and methanol (HPLC grade) were from Baker (Deventer, Netherlands) and cysteamine chloride, perchloric acid, trisodium citrate and 1,4-dithiothreitol were from Merck (Amsterdam, Netherlands).

2.2. Sample preparation

Plasma samples from patients were collected in cooled tubes, pretreated and analyzed immediately. As shown in Table 1, three different pretreatment procedures were performed, which allowed amifostine (procedure B), WR 1065 (procedure A) and the disulfides (procedure C) to be quantified. Unless mentioned otherwise, the procedures were performed at 0°C.

For the analysis of WR 1065, 1.0 ml of plasma

Table 1
Sample pretreatment



* Deproteinized plasma+WR 1729+buffer.

was added to 0.4 ml of 1 *M* $HClO_4$ in 2.7 m*M* EDTA in polypropylene test tubes, on ice, to precipitate the proteins. After centrifugation (15 000 *g*, 1 min) 750 μl of the supernatant (deproteinized plasma) was added to 50 μl of 24 μ*M* WR 1729 [in 0.1 *M* ClAcH–0.2 *M* $HClO_4$ (1:3, v/v) adjusted to pH 3.0 with sodium hydroxide] as an internal standard. 100 μl of an alkaline solution, containing 80 μl 0.5 *M* trisodium citrate buffer (brought to pH 3.0 with hydrochloric acid) and 20 μl of 5 *M* NaOH, was added to increase the pH of the sample to approximately 2.5, to prevent fast deterioration of the analytical column.

In order to determine the amifostine concentration, the plasma sample was deproteinized with $HClO_4$–EDTA as mentioned in the pretreatment procedure for WR 1065. The supernatant was incubated for 5 h at 37°C, to convert amifostine quantitatively into WR 1065. After addition of the internal standard and the citrate buffer–sodium hydroxide solution, the concentration of WR 1065, representing amifostine plus native WR 1065, could be determined in this sample.

For the analysis of the disulfides, 1.0 ml of plasma was added to 100 μl of 0.03 *M* dithiothreitol. The samples were incubated at room temperature for 15 min to convert the disulfides quantitatively into free WR 1065. Thereafter, the samples were further treated as mentioned for the analysis of WR 1065. The final concentration of WR 1065 determined in these samples represents the concentration of the disulfides plus native WR 1065.

Calibration standards of WR 1065, covering the concentration range of 0.15–12.5 μ*M*, were prepared on the day of use in blank deproteinized plasma (plasma after precipitation of the proteins with $HClO_4$–EDTA as mentioned in the pretreatment procedure for WR 1065). It was not possible to prepare the calibration standards in plasma because of the fast formation of disulfides after addition of WR 1065 to plasma. These calibration standards were used for the quantification of WR 1065 after the described procedures. For the analysis of the disulfides, 50 μl of 0.03 *M* dithiothreitol was added to 750 μl of each calibration standard, which corresponded to the amount of dithiothreitol added to each sample (Table 1, procedure C). This was necessary because of a possible overlap of the broad dithiothreitol peak at the beginning of the chromatogram with the peak of WR 1065 and the internal standard. The standard curves were constructed by linear regression (weight factor: $1/y$) of the peak height ratios WR 1065/WR 1729 plotted against the concentrations of the WR 1065 standards.

Quality control samples of amifostine and WR 1065 were prepared on the day of use. Because amifostine was not stable in plasma when stored for a longer period, stock solutions of amifostine were prepared in water at concentrations of 100, 30 and

7.5 μ*M* and stored in aliquots at −80°C. On the day of analysis, quality control samples were prepared by diluting these aliquots ten-fold with blank plasma, after which, they were treated as described in Table 1 (procedure B). Because WR 1065 is also not stable in plasma (rapid conversion into disulfides), stock solutions of WR 1065 were prepared in perchloric acid–monochloroacetic acid (3:1, v/v, adjusted to pH 3.0 with sodium hydroxide) at concentrations of 200, 50 and 5 μ*M* and stored in aliquots at −80°C. On the day of analysis, the quality control samples were prepared by twenty-fold dilution of these stock solutions in deproteinized plasma. They were further processed like the deproteinized plasma in scheme A of Table 1. The quality control samples of the disulfides were prepared by incubating plasma with WR 1065 at room temperature for 30 min at concentrations of 10, 2.5 and 0.25 μ*M*. The quality control samples were stored in aliquots at −80°C. On the day of analysis, the samples were thawed and treated according to procedure C in Table 1. These samples were also pretreated once according to the procedure described for the analysis of free WR 1065 (procedure A), to determine whether or not WR 1065 was quantitatively converted into disulfides.

2.3. Chromatography

The HPLC system consisted of a Promis II autosampler with a cooled tray (4°C), a Spectroflow 400 solvent delivery system, a degasser Model GT-103 (all from Separations, H.I. Ambacht, Netherlands) and a Decade wall jet electrochemical detector with a Au electrode (Antec, Leiden, Netherlands). For the acquisition and processing of the data, an Axxiom chromatography data system (Model 737) was used.

A 20-μl volume of the standards and samples was injected onto a Phenomenex Customsil 5 ODS-4 column (100×4.6 mm; Bester, Amstelveen, Netherlands) provided with a refillable guard column with Pellicular C_{18} refill (Alltech, Breda, Netherlands). The analytes were isocratically eluted with a mobile phase consisting of 40:60 (v/v) methanol–water containing 0.1 *M* monochloroacetic acid (adjusted to pH 3.5 with sodium hydroxide), 60 m*M* hexanesulfonic acid and 0.1 μ*M* cysteamine. The flow-rate was 1.0 ml/min and the temperature of the column was 36°C.

WR 1065 and WR 1729 were detected at +1.00 V vs. Ag–AgCl. Approximately 5 min before each analysis, the potential was set at −1.0 V for 5 s to "clean" the electrode surface. The total analysis time of one sample was 15 min.

2.4. Validation of the assay

The procedures developed for the quantitation of the three compounds were validated on six days by the duplicate analysis of the quality control samples to determine the between-day precision and accuracy and by the six-fold analysis of the quality control samples on one day to determine the within-day precision and accuracy. Calibration standards and quality control samples were analyzed in duplicate, starting with one measurement of each calibration standard and quality control sample, followed by the second measurement of each quality control sample and standard, in the reverse order. This order of sampling allows the detection of and correction for a possible systematic course in e.g. sample stability, detection response, etc.

The lower limit of quantitation was defined as the lowest concentration of WR 1065 on the standard curve that can be measured with acceptable accuracy and precision (<20%). This was determined by triplicate analysis of an extensive calibration curve in the low concentration range.

3. Results

3.1. WR 1065

In Fig. 2, the chromatograms are shown of blank plasma from a representative patient with and without the addition of WR 1065 and the internal standard, WR 1729. No interfering peaks were detected at the retention times of WR 1729 and WR 1065. The lower limit of quantitation of WR 1065 was 0.15 μ*M* in deproteinized plasma. The calibration curve was linear up to 12.5 μ*M*.

The validation parameters for the analysis of WR 1065 are shown in Table 2. The within-day and between-day coefficients of variation for WR 1065

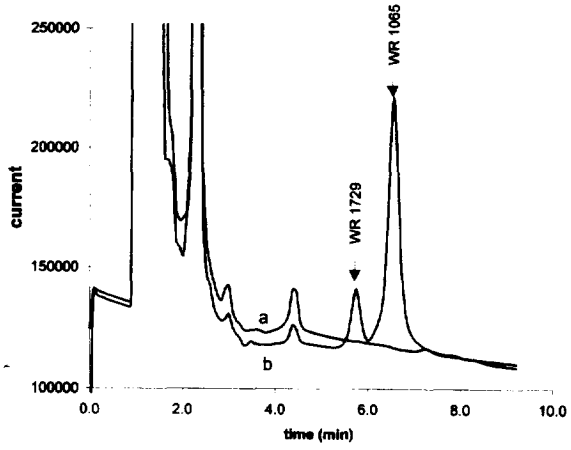


Fig. 2. Chromatograms of blank plasma from a representative patient (a) and spiked with 1.5 μ*M* internal standard, WR 1729, and 5 μ*M* WR 1065 (b).

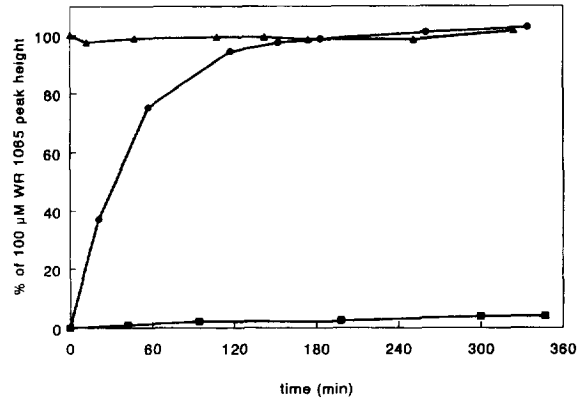


Fig. 3. WR 1065 concentrations expressed as % of peak height of 100 μ*M* WR 1065 peak when measuring: (a) stability of 100 μ*M* WR 1065 in acidic deproteinized plasma at 37°C (▲), (b) stability of 100 μ*M* amifostine in acidic deproteinized plasma at 0°C (■), (c) conversion of 100 μ*M* amifostine in acidic deproteinized plasma at 37°C (●).

were ≤4.4 and ≤8.2%, respectively. The within-day and between-day accuracy for WR 1065 ranged from 90.0 to 109.8% and from 97.6 to 101.5%, respectively (Table 2).

Stock solutions of WR 1065 in ClAcH–$HClO_4$ were stable at −80°C for at least six months. In deproteinized plasma, WR 1065 was stable for at least 7 h at a temperature of 37°C (Fig. 3a) and for more than 10 h at 25°C.

Table 2
Validation parameters of amifostine, WR 1065 and disulfides in plasma

Quality control sample (μM)	Within-day (n=6)		Between-day (n=6)	
	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)
WR 1065				
0.25	4.4	90.0	8.2	101.5
2.50	1.6	97.2	5.0	99.2
10.00	3.1	109.8	7.7	97.6
Amifostine				
0.75	4.7	110.7	13.1	101.5
3.00	3.1	88.3	3.0	99.7
10.00	4.9	100.8	6.9	99.4
Disulfides				
1.00	8.5	110.2	4.5	104.9
3.00	2.3	99.2	5.0	104.0
10.00	0.7	103.6	5.5	103.3

3.2. Amifostine

To quantify amifostine, it should be quantitatively converted into WR 1065 during the pretreatment procedure. This conversion takes place in acidic deproteinized plasma and is dependent on the temperature. At 37°C, amifostine was completely converted to WR 1065 in approximately 3 h (Fig. 3c). When the sample was maintained at 0°C, only 6% conversion occurred after 6 h (Fig. 3b).

The within-day and between-day coefficients of variation for amifostine were ≤4.9 and ≤13.1%, respectively. The within-day and between-day accuracy for amifostine ranged from 88.3 to 110.7% and 99.4 to 101.5%, respectively (Table 2). Stock solutions of amifostine in water, used for the preparation of the quality control (QC) samples, were stable for at least three months, when stored at −80°C.

3.3. Disulfides

The QC samples of the disulfides were prepared by the addition of WR 1065 to plasma. The quantitative conversion of WR 1065 to the disulfides was checked by measuring the amount of free WR 1065 in representative QC samples. No WR 1065 could be

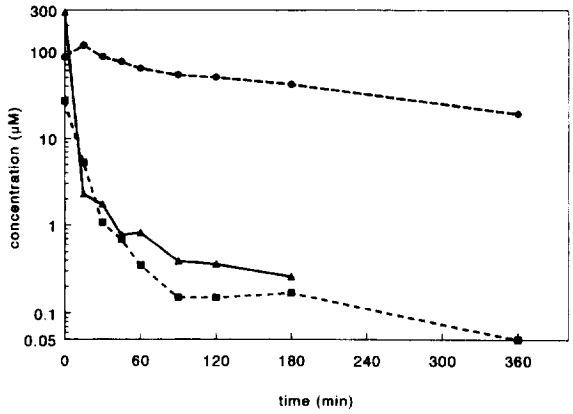


Fig. 4. Plasma concentration–time curves of amifostine (▲), WR 1065 (■) and the disulfides (●) in a representative cancer patient after treatment with 740 mg/m^2 amifostine as a 15-min i.v. infusion.

detected, indicating that all WR 1065 was converted to (mixed) disulfides.

In Table 2, the validation parameters for the analysis of the disulfides are shown. The within-day and between-day precision for the disulfides were ≤8.5 and ≤5.5%, respectively. The within-day and between-day accuracy for WR 1065 ranged from 99.2 to 110.2% and 103.3 to 104.9%, respectively. The QC samples of the disulfides were stable at −80°C for more than five months.

3.4. Samples from a patient

The plasma concentration–time curves of amifostine, WR 1065 and the disulfides from a patient treated with 740 mg/m^2 amifostine as a 15-min i.v. infusion are shown in Fig. 4. Concentrations of amifostine, WR 1065 and the disulfides could be measured for up to 3, 6 and 24 h, respectively. Samples taken at the early time points had to be diluted to obtain a concentration that was within the linear range of the calibration curve.

4. Discussion

To study the pharmacokinetics of amifostine, a fast and sensitive assay for the combined analysis of amifostine and its main metabolites, WR 1065 and the (mixed) disulfides, is desired. Until now, the reported assays were mostly restricted to the analysis of only one or two of these compounds, whereas, in our assay, all three compounds could be measured with one method.

4.1. WR 1065

We developed a very sensitive detection method for WR 1065. A lower limit of quantitation of 0.15 μ*M* could be obtained by electrochemical detection with a wall-jet gold electrode. The highest sensitivity was obtained at a potential of +1.0 V. Although many endogenous compounds were oxidized at this potential, optimization of the chromatographic conditions resulted in a good separation of WR 1065, the internal standard and endogenous compounds. The sensitivity of our method was comparable with, or higher than, that of earlier reported methods. For these methods, in which a mercury/gold electrochemical detector was used, lower limits of quantitation of 2.5 [11] and 0.25 μ*M* [12] were reported. The accuracy and precision of our method were comparable to those of the reported methods (<10%). The disadvantage of the mercury/gold detector, as was used in the literature, was the maintenance of the electrode. Every two weeks, the electrode had to be recoated, followed by overnight equilibration [8], whereas our electrode had only to be cleaned with acetone before each run and was equilibrated within 30 min. In order to prevent a decrease in the detection signal by pollution of the electrode surface, a "clean up" pulse was programmed before each analysis. The potential was set to −1.0 V for 5 s. After this pulse, it takes approximately 6 min before the electrode is equilibrated. Nevertheless, including this equilibration period, the analysis time was only 15 min per sample. For these reasons, our detection method was preferred above the reported methods.

The pretreatment procedure for the analysis of WR 1065 is mandatory. Shaw et al. [11] reported that immediate deproteinization of the samples with acid was necessary to prevent the formation of disulfides, because a low pH value minimizes oxidation of sulfhydryl compounds. By the addition of $HClO_4$–EDTA to a plasma sample, immediately after the addition of WR 1065 to the plasma, good recovery was obtained [11]. We used the same pretreatment method as Shaw et al. [11]. In the case of plasma

samples, measurements of WR 1065 will often be made in the presence of amifostine. Therefore, it is important that no hydrolysis of amifostine to WR 1065 takes place after the addition of perchloric acid. According to Shaw et al. [11], this hydrolysis is strongly dependent on the pH and the temperature. We investigated this hydrolysis in deproteinized plasma at 0 and 37°C. It can be concluded from the results that for an accurate measurement of the WR 1065 concentration, it is important to maintain the sample at 0°C prior to analysis, which should occur as soon as possible after collecting the sample from the patient.

We could not determine the recovery of WR 1065 from plasma by our method because of the fast formation of disulfides after addition of WR 1065 to the plasma. However, the high accuracies (99–110%) for the disulfides after the same deproteinization procedure as used for the analysis of WR 1065 in plasma samples indicate that no loss occurred during the precipitation step. These results and the good precision and accuracy of the analysis of WR 1065 in deproteinized plasma indicate that this method is suitable for the analysis of WR 1065 in plasma samples from patients.

4.2. Amifostine

We were able to analyze amifostine by its conversion to WR 1065 during the pretreatment and the subsequent measurement of the WR 1065 concentration. The conversion was based on the hydrolysis of amifostine in acidic medium at 37°C. After subtraction of the native WR 1065 concentration, the amifostine concentration could be calculated. It can be concluded from the high accuracies (88–110%) that a high recovery rate was obtained using our procedure. The sensitivity of this analysis is the same as that shown for the analysis of WR 1065, because it is based on the detection of WR 1065.

Direct measurement of amifostine was also possible with electrochemical detection on a mercury/gold electrode [10]. However, this method was not as sensitive (lower limit of quantitation, 1 μM) as our method. Because WR 1065 could also be detected with this electrode, it was possible to combine these assays. However, the different chromatographic behaviours of the two compounds made it necessary to change the mobile phase after the elution of amifostine, which, nevertheless, resulted in a long retention time (20 min) for WR 1065 [10]. Swynnerton et al. [6] described a method for the fluorimetric detection of amifostine after precolumn derivatization with fluorescamine. Although this method was rather sensitive (lower limit of quantitation=0.25 μM), the run time for one chromatogram (50 min) was very long. Furthermore, this method could not be easily combined with the analysis of WR 1065 or the disulfides. Therefore, our method was preferred over the assays described in the literature.

4.3. Disulfides

A method for the analysis of the symmetrical disulfide, WR 33278, was described by Shaw et al. [14]. WR 33278 could be detected with a dual mercury/gold electrode by reduction to WR 1065 at −1.0 V on the upstream electrode and subsequent oxidation of WR 1065 at +0.15 V on the downstream electrode. This sensitive method (0.2 μM) was accurate and precise and might be combined with the analysis of WR 1065. A drawback of the procedure is that only the symmetrical disulfide could be quantified. However, when the total amount of disulfides serve as an exchangeable pool of WR 1065, it is clinically more relevant to measure the total amount of disulfides instead of the symmetrical disulfide alone. Two methods were described for analysis of the total amount of disulfides by reduction of the disulfide bonds with *n*-butylphosphine or dithiothreitol and subsequent determination of the WR 1065 concentration [7,13]. In our method, we also used dithiothreitol to reduce the disulfide bonds. With this procedure, we obtained high accuracies of 99–110%, whereas the sensitivity was the same as that shown for the analysis of WR 1065.

The developed method was used for the measurement of amifostine, WR 1065 and the disulfides in the plasma from a representative patient treated with 740 mg/m^2 amifostine as a 15-min infusion. The sensitivity of our assay made it possible to monitor amifostine, WR 1065 and the disulfides in plasma for at least 3, 6 and 24 h, respectively.

It can be concluded that our assay for the combined analysis of amifostine, WR 1065 and disulfides in plasma proved to be accurate, precise and simple.

As an improvement to earlier described methods, our assay was very suitable for pharmacokinetic studies, because of its sensitivity and the relatively fast and simple analysis of amifostine and its main metabolites in one system.

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