Development and Validation of an HPLC Method for Determination of Amifostine and/or Its Metabolite (WR-1065) in Human Plasma Using OPA Derivatization and UV Detection

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Abstract

A rapid, sensitive and reproducible HPLC method was developed and validated for the analysis of amifostine (AMF) and/or its metabolite, WR-1065 in human plasma. The method involves the alkylation of free sulfydryl group with iodoacetic acid followed by derivatization of the drug and its metabolite with o-phthaldialdehyde (OPA) and UV detection at 340 nm. The derivatized AMF and WR-1065 were eluted in less than 11 min, and in the case of the metabolite with no interferences from the endogenous plasma peaks. Cystein was used as the internal standard. Analysis was carried out on a Eurosphere Performance (RP-18e, 100 × 4.6 mm) analytical column. The mobile phase was a mixture of methanol and phosphate buffer 0.03 M pH = 2.7 at a ratio of 40: 60v/v, respectively, with a flow rate of 1.5 mL min\textsuperscript{-1}. Limit of detection was 0.5 µgmL\textsuperscript{-1}. The method involved a simple extraction procedure for AMF and/or its metabolite and analytical recovery was 90 ± 0.9%. The calibration curve was linear over the concentration range of 1-200 µgmL\textsuperscript{-1}. The coefficients of variation for intra-day and inter-day assays were less than 10%.

Keywords: amifostine; WR-1065; human plasma; HPLC; OPA derivatization.

Introduction

Amifostine (2-(3-aminopropylamino) ethylsulfanyl phosphonic acid, WR-2721) (AMF), a synthetic aminothiol compound, is used as a cytoprotective agent in cancer in radiotherapy and chemotherapy involving DNA-binding chemotherapeutic agents. As a prodrug, it is dephosphorylated in the tissue by alkaline phosphatase, to its active free thiol metabolite, WR-1065 (2-(3-aminopropylaminoethanethiol) (Figure 1). WR-1065 is able to scavenge free radicals, to deplete oxygen and covalently bind to active metabolites of antineoplastic agents. The sulfur-hydrogen bond of WR-1065 can easily donate its hydrogen ion to radiation-induced free radicals and hydrated electrons that can damage DNA, thereby decreasing cell damage [1].

WR-1065 exists in human plasma in various forms; only trace amounts [~1%] are in reduced (sulfydryl) form, the remaining part is oxidized and exists as various disulfides. About 50% of WR-1065 is bound to proteins in plasma (via disulfide bond), whereas the remaining part exists as free disulfides, mostly as WR-33287[2] (Figure 1).

There is no universal technique for determination of both AMF and its major metabolite WR-1065 in plasma. The
A rapid and sensitive and accurate HPLC method based on alkylation of free sulfydryl group with iodoacetic acid of AMF and/or its major metabolite, WR-1065, and derivatization of analytes with o-phthaldialdehyde (OPA) followed by UV detection (Figure 2). The method enables determination of the drug alone for in-vitro studies. Also, the method is applicable for simultaneously determination of AMF and its metabolite, with an acceptable accuracy at drug concentrations as low as 0.5 µg/mL in plasma using a simple extraction procedure. The proposed assay method needs small sample volume (100 µL) [14-15]. Separation was performed on a reversed-phase
Eurospir column and therefore, it allows easy optimization of the chromatographic conditions to obtain desirable resolution in a reasonable time, without interfering with endogenous plasma peaks. The sample preparation only involves a simple extraction procedure and no evaporation step is required.

It is necessary to mention that AMF has very short half-life (10 min) and rapidly convert to WR-1065. Thus, validation assessment of the proposed method was based on determination of the metabolite, WR-1065 [12].

**Experimental**

**Chemicals**

AMF was supplied by ZiboPaxinPharm Chemical Company (China), dihydrochloride salt of WR-1065, OPA and 2-mercaptoethanol (2-ME) were obtained from Sigma- Aldrich company (France). L-cysteine, iodoaceticacid (IAA), HPLC-grade methanol, and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was prepared by double distillation and purified additionally with a Milli-Q system.

**Chromatographic conditions**

HPLC system consisted of a model Manager 5000, a model Pump 1000, and a model D-14163 injector connected to a model PDA detector 2800, all from Knauer (Berlin, Germany). The separation was performed on Eurospher Performance (RP-18e, 100 × 4.6 mm) column from Knauer (Berlin, Germany).

An isocratic elution system was employed with mobile phase consisted of methanol and 0.03M phosphate buffer (40:60 v/v, respectively) adjusted to pH = 2.7 with a flow rate of 1.5 mLmin⁻¹.

Chromatograms were monitored by diode array detection (200-400 nm) and displayed at single wavelength of 340 nm. The mobile phase was prepared daily and degassed by ultrasonication before use. The mobile phase was not allowed to recirculate during the analysis.

**Standard solutions**

A stock solution (0.1 mgmL⁻¹) of AMF was prepared in 0.05 M phosphate buffer pH = 6.4. Then, standard solutions were prepared at concentrations of 100, 90, 70, 50, 30, 10 and 1 µgmL⁻¹ by serial dilution. Also, a stock solution of WR-1065 were prepared in 0.05 M HCl solution.

Then, standard solutions were prepared at concentrations of 10, 50, 100, 200, 500, 1000 and 2000 µgmL⁻¹ of WR-1065 in 0.05 M HCl solution and stored at -20°C until further use.

The internal standard (cysteine) solution was prepared by dissolving 3 mg cysteine in 10 mL 0.05 M HCl to obtain a concentration of 300µgmL⁻¹ and stored at 4°C until further use.

**Plasma sample preparation**

To 100 µL of plasma in a polyethylene tube were added 20 µL of cysteine solution as internal standard (at a final concentration of 30 µgmL⁻¹), 40 µL WR-1065 and 20 µL 2-mercaptoethanol.

The sample was mixed and after incubation for 30 s at room temperature plasma proteins were removed by precipitation with 200 µL acetonitrile followed by centrifugation at 12000 g for 10 min. Then 40µL of supernatant was mixed with 100µL of IAA solution (0.8M in 0.1 M sodium borate buffer pH 10.5) and 240 µL of 0.1 M sodium borate buffer (pH 11.5). After incubation for 30 s at room temperature 40 µL of OPA-2-ME reagent was added and after 3 min 100 µL of reaction mixture was injected into the HPLC system.

**Stability**

The stability of WR-1065 was assessed for spiked plasma samples stored at −20°C for up to one week, and at ambient temperature for at least 24 h. The stability of each stock solution stored at 20°C was determined for up to 4 weeks by injecting appropriate dilution of the stock in 0.05HCl on day 1, 15 and 30 and comparing their peak areas with freshly prepared solution on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

**Plasma standard curve**

Blank plasma was prepared from heparinized whole-blood samples, collected from healthy volunteers and stored at 20°C. After thawing, 40 µL of one of the above-mentioned WR-
1065 working standards were added to yield final concentrations of 1, 5, 20, 50, 100 and 200 µgmL\(^{-1}\). Internal standard solution was added to each of these samples to yield a concentration of 30 µgmL\(^{-1}\). The samples were then prepared for analysis as described above. Calibration curves were constructed by plotting peak area ratio (y) of WR-1065 to the internal standard versus WR-1065 concentrations (x). A linear regression method was used for quantitation.

**Precision and accuracy**

The precision and accuracy of the method were examined by adding known amounts of WR-1065 to pool plasma (quality control samples). For intra-day precision and accuracy, six replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days.

**Limit of quantification (LOQ) and recovery**

The analytical recovery for plasma at three different concentrations of WR-1065 (5, 50 and 200 µgmL\(^{-1}\)) was determined. Known amounts of WR-1065 were added to drug-free plasma and the internal standard was then added. The relative recovery of WR-1065 was calculated by comparing the peak areas for extracted WR-1065 from spiked plasma and a standard solution of WR-1065 in 0.05 M HCl containing internal standard with the same initial concentration (six samples for each concentration level).

**Selectivity and specificity**

Control human plasma, obtained from three healthy volunteers, was assessed by the procedure as described above and compared with respective plasma samples to evaluate selectivity and specificity of the method.

**Results and Discussion**

**Method development**

AMF and WR-1065 are primary amines and OPA is a suitable and selective derivatizing reagent for both compounds. Also, cysteine is structurally similar to WR-1065 (i.e. both are
The average regression equation was found by applying the least squares method and calculated as \( Y = 0.0211X \). The relative standard deviation for the slope was 1.1\% and the average correlation coefficient was 0.993.

The repeatability of derivatization reaction was evaluated by calculating relative standard deviation (RSD) values of six consecutive injections of the working standard solutions. The RSD of peak area of WR-1065 was less than 10\%.

For the sample preparation, several tedious extraction methods have been used for analysis of WR-1065 in biological fluids [5–9]. In addition, some of the methods involved using large volumes of plasma samples or toxic extraction agents which limited their application to analyze large numbers of clinical samples [3–5]. In our method, sample preparation involves simple extraction procedure and no evaporation step is required.

The analytical recovery for plasma at three different concentrations of WR-1065 was determined. Known amounts of WR-1065 were added to drug-free plasma in concentrations ranging from 1–200 \( \mu g\text{mL}^{-1} \). The internal standard was added and the absolute recovery of WR-1065 was calculated by comparing the peak areas of extracted WR-1065 from spiked plasma and a standard solution of WR-1065 containing internal standard with the same initial concentration.

As shown in Table 1 the average recovery of WR-1065 from plasma, determined at three different concentrations of the metabolite (5, 50, 200 \( \mu g\text{mL}^{-1} \)), was 90 ± 0.9\% (\( n = 6 \)).

Using OPA dervatization and UV detection, the limit of quantification (LOQ) was calculated as 0.5 \( \mu g\text{mL}^{-1} \) for WR-1065, which is sensitive enough for drug monitoring and for pharmacokinetic studies.

Validation of the method

A linear relationship between the absorbance at 340 nm and the concentration of WR-1065 was established over the examined concentration range (1-200 \( \mu g\text{mL}^{-1} \)).
applied conditions. The proposed method is well suited for routine application in the clinical laboratories because of the speed of analysis and simple extraction procedure. No change in the column efficiency and back pressure was also observed over the entire study time, proving its suitability.

Conclusion

A robust and sensitive HPLC method has been described for analysis of WR-1065, as an active and major metabolite of amifostine, in human plasma. Derivatization with OPA is an efficient method for enhancing chromatographic detection of amifostine and other structurally related compounds such as WR-1065. In addition, the use of a simple procedure for sample preparation makes the proposed method a fast and reliable one for pharmacokinetic studies of WR-1065 in humans.

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