High-performance liquid chromatographic analysis of the new antitumour drug SK&F 104864-A (NSC 609699) in plasma*

J.H. BEIJNEN,t S. RODENHUIS§ and W.J.M. UNDERBERG**

Abstract: A high-performance liquid chromatographic (HPLC) assay is described for the determination of the new, investigational antitumour drug SK&F 104864-A and its lactone ring opened form (SK&F 105992). The analytical methodology reported here involves a protein precipitation step with methanol as sample pretreatment procedure. The instability of the drug necessitates that the plasma fraction is obtained within 5 min after blood sampling by centrifugation, immediately followed by protein precipitation with cold methanol (-30°C). The methanolic extract can be stored at -30°C for several days without deterioration of the analytes. Stability data of the drug and its lactone ring opened metabolite in plasma and after methanolic extraction are discussed. The parent drug and the metabolite are separated by reversed-phase ion-pair liquid chromatography on a LiChrosorb RP-18 column, using methanol–water eluent (pH 6.0) with sodium dioctylsulphosuccinate (DOSS) as ion-pairing agent and fluorescence detection. The proposed method has been validated and, subsequently, implemented in a phase I clinical trial for pharmacokinetic evaluation of the new cytotoxic agent.

Keywords: SK&F 104864-A (NSC 609669), SK&F 105992, HPLC, stability, bio-analysis.

Introduction

SK&F 104864-A, [S]-9-dimethylaminomethyl-10-hydroxy-camptothecin (NSC 609669) (I), see Fig. 1, is a novel, investigational, antitumour drug which has been developed by Smith Kline and French Laboratories. The drug is a semisynthetic analogue of camptothecin (NSC 100880) which showed promising responses in earlier phase I trials [1, 2]. The mechanism of action of this class of compounds is attributed to inhibition of DNA topoisomerase I. Unfortunately, clinical evaluation of camptothecin had to be discontinued due to its toxicity profile including myelosuppression, gastrointestinal toxicity and haemorrhagic

![Figure 1](https://example.com/fig1.png)

Chemical structures and equilibrium reactions between I (SK&F 104864-A) and II (SK&F 105992).

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† Author to whom correspondence should be addressed.
In comparison with camptothecin, I is more water soluble, due to introduction of the basic side chain, and preclinical investigations indicate that it is likely to be less toxic with a high degree of activity against solid tumours and also in multi-drug resistant cell lines [4]. Furthermore, the compound appears to be a more selective inhibitor of topoisomerase I than camptothecin. These data rationalize the phase I clinical trial with pharmacokinetic evaluation.

I is not stable at physiological pH and equilibrium processes favour hydrolysis of the lactone ring to yield the hydroxy-acid SK&F 105992 (II) (Fig. 1). Only the closed lactone form of the drug inhibits topoisomerase I. Hydrolysis of the parent drug will have a major impact on the pharmacokinetic interpretation and, therefore, a bio-analytical method is required which is capable of determining the drug and its hydrolysed form selectively and preferably in one run.

This paper is the first report in the literature on the bioanalysis of I and the ring opened form of the drug.

**Experimental**

**Materials**

SK&F 104864-A (I) originated from Smith Kline and Beecham Laboratories, USA. SK&F 105992 (II) was prepared by adding 40 μl of 1 M NaOH solution to 1 ml stock solution of I in methanol (25°C). This procedure gives an almost quantitative conversion of I into II, instantaneously, as verified by HPLC. This solution of II is stable for at least 7 days at 4°C. All reagents were of analytical grade and double-distilled water was used throughout.

**Chromatography**

The chromatographic system consisted of a solvent delivery system, type 6000 A (Waters Assoc. Milford, MA, USA) and an automatic sample injection device model SP 8880 (Spectra Physics, Santa Clara, CA, USA). Separation was performed on a LiChrosorb RP-18 (particle size: 5 μm) column (125 × 4 mm i.d., Merck, Darmstadt, FRG). To protect the analytical column, a pre-column (20 × 4 mm i.d.), dry-packed with Bondapak Corasil C18 (particle size: 37-50 μm) material (Waters Assoc.), was used. The pre-column was replaced weekly. The mobile phase consisted of 325 ml methanol, 215 ml water, 20 ml 0.25 M sodium dioctylsulphosuccinate (DOSS) solution, 11.5 ml 1.0 M phosphate buffer pH 6.0 and 1.5 ml triethylamine; the apparent pH of this mixture is adjusted to 6.0 with phosphoric acid. The flow rate was maintained at 1.0 ml min⁻¹. The column effluent was monitored spectrofluorimetrically using a Perkin-Elmer LS 40 fluorescence detector with the excitation wavelength set at 381 nm and emission wavelength at 527 nm. Retention times and peak areas were measured with an SP-4290 (Spectra Physics) data system.

**Sample pretreatment**

For the isolation of I and II from plasma a 100-μl sample is mixed with 400 μl cold methanol (−30°C). After vortex mixing for 10 s and centrifugation at 12,000 rpm for 2 min the clear supernatant is transferred into a glass autosampler vial and stored, immediately, at −30°C, prior to analysis. For the analysis, the sample is taken from the freezer and an aliquot of 20 μl (for the 10-1000 ng ml⁻¹ range) or 100 μl (for the 1-10 ng ml⁻¹ range) is injected, directly, onto the HPLC column.

**Calibration**

Blank plasma samples (100 μl), in polypropylene Eppendorf tubes, are spiked with 10-100 μl of an appropriate amount of I or II in methanol. Cold methanol (−30°C) is added up to a total volume of 500 μl. The samples are further treated as described under Sample pretreatment. Blank plasma samples of each patient are utilized for its own calibration.

**Validation**

Recoveries of I and II from plasma have been calculated by comparison with aqueous solutions with known quantities of I and II. Linearity was investigated by analysing spiked plasma samples (see Calibration). Precision and accuracy were determined on different days. For the establishment of the detection limit of the HPLC assay the fluorescence detector was set at a response equal to three times the average noise level.

**Pharmacokinetics**

A 67-year-old patient suffering from gall bladder cancer was treated with 4.1 mg (2.5 mg m⁻²) of I dissolved in 500 ml 0.9% sodium chloride by continuous infusion over 24 h. Periodically, blood samples were
collected from an indwelling i.v. cannula placed in the arm contralateral to that receiving the drug. Samples were taken in heparinized tubes (10 IU ml\(^{-1}\)) prior to the start of the infusion, during infusion, at the end of the infusion and at specific times post-infusion. Plasma was immediately isolated by centrifugation and treated and stored as described under **Sample pretreatment**.

**Results and Discussion**

**Chromatography**

Several chromatographic systems have been tested with the purpose to find the optimal conditions for analysing \(I\) and \(II\). Straight phase chromatography on silica columns appeared not to be suitable due to the highly polar character of \(I\) and \(II\). On the other hand, reversed-phase HPLC using a C18 column gave acceptable chromatographic results. The influences of each component of the mobile phase on the chromatographic behaviour of the analytes was studied by omitting each of them, in turn. These experiments revealed the relevance of the presence of each component. The pH of the eluent was chosen in view of the rate of reversible reactions between \(I\) and \(II\). At pH 6 the rate of the conversion of \(I\) into \(II\) is low, whereas the reverse reaction turns out to be pH independent [5]. At pH 6.0 the basic side chain of \(I\) is expected to be protonated giving ion pairs with DOSS in the eluent resulting in increased retention. Triethylammonium ions act as tail reducer.

\(I\) and \(II\) show native fluorescence, very suitable for sensitive and selective detection. The selection of the wavelengths was based on scanning experiments of the analytes in the mobile phase for maximum fluorescence intensities.

**Sample pretreatment**

The sample pretreatment procedure for isolation of \(I\) and \(II\) from the biological matrix must be executed in such a way that the reversible reaction between \(I\) and \(II\) is slow and that the results give a proper presentation of the *in vivo* situation of \(I\) and \(II\). From the stability experiments it may be concluded that the reversible reaction is manageable at physiological pH. At that pH, \(I\) probably exists as a cation (protonated amino side cation) and \(II\) as a zwitterionic species (protonated amino side chain and deprotonated carboxylic function) which hampers effective isolation by liquid–liquid extraction and liquid–solid extraction; these sample pretreatment techniques have been investigated and gave only low recoveries for \(I\) and \(II\). On the other hand, a protein precipitation as sample pretreatment procedure appeared suitable. The method has the advantage that it can be performed quickly and the extract contains methanol which stabilizes the analytes. A disadvantage is that the sample is diluted.

**Chemical stability of \(I\) and \(II\)**

Stock solutions of \(I\) in methanol (concentration range: 10 ng ml\(^{-1}\) up to 1 mg ml\(^{-1}\)) are stable for at least 1 month when stored in the refrigerator. However, aqueous solutions of \(I\) are not stable [5]. The unstable part of the molecule is the lactone ring which is opened at physiological pH yielding \(II\) (Fig. 1). This is a reversible reaction and the equilibrium situation depends upon the pH of the medium. At pH >7 the equilibrium process favours the ring opened form and in acidic solution the reverse reaction with the formation of \(I\). From the experiments on the reverse reaction of \(I \rightarrow II\) it appeared that the total fluorescence intensity, in terms of peak area units from the integrator, remained constant indicating that \(I\) and \(II\) have identical fluorescence intensities at the analytical wavelengths.

The chemical stability of \(I\) in plasma (pH = 8; concentration 500 ng ml\(^{-1}\)) at room temperature was investigated. The drug concentrations as function of time were: \(t = 0\), \(I = 100\%\); \(t = 10\) min, \(I = 93\%\), \(II = 7\%\); \(t = 50\) min, \(I = 53\%\), \(II = 47\%\); and \(t = 120\) min, \(I = 20\%\), \(II = 80\%\). The equilibrium is reached after 200 min, whereby \(I = 5\%\) and \(II = 95\%\). No other products were noticed in the chromatograms and the sum of fluorescence yields of \(I\) and \(II\) remained constant during the (pre-) equilibrium situation. This demonstrates that it is important to process the plasma fraction as quickly as possible after sampling. No ways were found to stabilize the analytes in plasma except for a quick isolation from plasma. When the plasma fraction is deproteinized with cold methanol, the resulting extract is stable for at least 7 days at \(-30^\circ\text{C}\). This has been investigated with methanolic extracts from plasma samples with initial concentrations 90% \(I\), 10% \(II\); 50% \(I\) and 50% \(II\); 10% \(I\) and 90% \(II\), with the same results and justifies the proposed working method as described under **Sample**
When the methanolic extracts are stored at 4°C, 10% of the initial concentration of I is found to be converted into II within 60 min.

Validation of the assay

The analytical methodology has been validated in terms of recovery, precision, accuracy and linearity for the parent drug and the lactone ring opened form. The results of these experiments are shown in Tables 1–3. A structural analogue of I, SK&F 105107 also possessing a lactone ring, was available, but the use of this compound as internal standard offered no advantages and, therefore, was omitted in further studies. Furthermore, the use of an internal standard with an unstable lactone ring might be expected to introduce an extra uncertainty factor.

The detection limit of the assay is 0.2 ng ml\(^{-1}\) for I as well as for II using only a 100-μl plasma sample. Blank samples show an interfering plasma peak with almost the same retention time as II, see Fig. 2. Interference occurs when concentrations lower than 10 ng ml\(^{-1}\) of II have to be measured. However, the interfering peak appears to be very reproducible per patient and II can be measured very accurately when the chromatograms are reprocessed with subtraction of the interfering peak. This method gives reliable results, as shown in Tables 1–3.

Plasma concentrations of I and II from the first patient treated with a very low dose of I, the first dose step in the phase I study, given by a 24-h infusion regimen, are depicted in Fig. 3 and demonstrate the applicability of the assay for pharmacokinetic evaluation of the drug.

### Table 1
Recovery of I and II from plasma

<table>
<thead>
<tr>
<th>Concentration (ng ml(^{-1}))</th>
<th>Recovery I (%)</th>
<th>n</th>
<th>Recovery II (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>101.1 ± 4.0</td>
<td>5</td>
<td>105.6 ± 5.9</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>100.8 ± 4.2</td>
<td>5</td>
<td>93.5 ± 3.8</td>
<td>5</td>
</tr>
<tr>
<td>500</td>
<td>101.3 ± 2.7</td>
<td>4</td>
<td>93.1 ± 4.8</td>
<td>5</td>
</tr>
</tbody>
</table>

### Table 2
Equations of calibration lines for the analysis of I and II in plasma

**I**

1–10 ng ml\(^{-1}\); \(y^I = 19.21 (±0.44) x - 1.90 (±3.66)\) \(r^2 = 0.9979 \ n = 6\)

10–100 ng ml\(^{-1}\); \(y^I = 2.04 (±0.08) x - 6.57 (±6.46)\) \(r^2 = 0.9943 \ n = 6\)

100–1000 ng ml\(^{-1}\); \(y^I = 0.17 (±0.001) x - 1.88 (±1.44)\) \(r^2 = 0.9996\) \(n = 6\)

**II**

1–10 ng ml\(^{-1}\); \(y^II = 25.95 (±1.01) x - 8.33 (±8.40)\) \(r^2 = 0.9939 \ n = 6\)

10–100 ng ml\(^{-1}\); \(y^II = 2.32 (±0.07) x - 7.93 (±6.26)\) \(r^2 = 0.9958 \ n = 6\)

100–1000 ng ml\(^{-1}\); \(y^II = 0.39 (±0.01) x - 3.91 (±6.34)\) \(r^2 = 0.9967 \ n = 5\)

*Where x is the concentration of I in ng ml\(^{-1}\) and \(y^I\) and \(y^II\) are integrator peak height units and \(y^II\) peak area units.

†Where x is the concentration of II in ng ml\(^{-1}\) and \(y^I\), \(y^II\) are integrator peak height units and \(y^II\) is in peak area units.

### Table 3
Accuracy and precision for the bio-analysis of I and II

<table>
<thead>
<tr>
<th>Theoretical conc. (ng ml(^{-1}))</th>
<th>Measured conc. (ng ml(^{-1}))</th>
<th>Accuracy (%)</th>
<th>RSD (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>97.5</td>
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<td>5</td>
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<td>50</td>
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<td>100.4</td>
<td>0.9</td>
<td>5</td>
</tr>
<tr>
<td>500</td>
<td>508.5</td>
<td>101.7</td>
<td>0.9</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>5</td>
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<td>100.0</td>
<td>5.5</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>53.3</td>
<td>106.6</td>
<td>2.8</td>
<td>5</td>
</tr>
<tr>
<td>500</td>
<td>502.9</td>
<td>100.5</td>
<td>2.9</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 2
Chromatograms of blank plasma sample (A), plasma sample spiked with I (10 ng ml$^{-1}$) (B) and plasma sample spiked with II (10 ng ml$^{-1}$) (C) after reprocessing procedure with subtraction of blank value. Experimental conditions as described in the text.

Figure 3
Plasma concentration time curves of I (■) and II (□). Patient treated with 2.5 mg m$^{-2}$ (total dose: 4.1 mg) of I by continuous infusion over 24 h.
In conclusion, a simple, highly sensitive and validated HPLC method for the analysis of the new investigational cytotoxic drug SK&F 104864-A and its hydrolysed form in plasma has been developed which can be used for phase I pharmacokinetic studies.

References


