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# New protamine quantification method in microtiter plates using *o*-phthaldialdehyde/*N*-acetyl-L-cysteine reagent

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**Abstract**

Protamine is a well-known excipient in pharmaceutics. It represents a peptide consisting of exclusive aliphatic amino acids, hence it cannot be quantified by UV-spectroscopy ($\lambda_{max}$ 280 nm). A new and sensitive quantification method based on the derivatisation of protamine with *ortho*-phthaldialdehyde (OPA) in the presents of 2-mercaptoethanol (ME) or *N*-acetyl-L-cysteine (NAC) in basic aqueous solution using 96-well microtiter plates are introduced in this report. The resulting isoindol derivatives reveal a fluorescence excitation (maximum $\lambda_{ex}$ 345 nm) and emission (maximum $\lambda_{em}$ 450 nm) spectra. Derivatives of OPA/NAC reagent were found to be useful for protamine quantification in pharmaceutical nanoparticle preparation containing DNA. A sufficient stability of the isoindol derivatives was shown. It was possible to determine protamine free base, protamine sulphate and protamine chloride with limits of detection less than 1.1 μg/ml.


*Keywords:* Protamine; OPA/NAC reagent; Quantification; Microtiter plate; *Ortho*-phthaldialdehyde; *N*-acetyl-L-cysteine

## 1. Introduction

Protamines are strongly cationic peptides with a molecular mass of approximately 6000 Da found in the spermatic cells of vertebrates and cephalopods. For pharmaceutical use the protamine or its salts are obtained from salmon sperm (Ando et al., 1973). Besides its use of prolongation of insulin release in the diabetes mellitus therapy for the last decades and as an antidote for heparin intoxication in haematology, protamine became in the last few years a promising agent in gene therapy for enhancing the transfection of genes with lipoplexes (Boulikas and Martin, 1997; Sorgi et al., 1997) and the delivery of antisense oligonucleotides using nanoparticles (Gonzalez Ferreiro et al., 2001; Junghans et al., 2000). For this reason it is essential to get an information about the content of protamine in a drug formulation in pharmaceutics.

The sequences of protamine containing only aliphatic amino acids, thus the standard method of protein quantification by measuring the UV-absorption at $\lambda_{max}$ 280 nm of the aromatic amino acids of the peptides or proteins is not applicable (Okamoto et al., 1993).

Colorimetric spectrometry and measuring the absorption at $\lambda_{max}$ 500 nm after Sakaguchi reaction (Weber, 1929) is the commonly used method to quantify protamine in aqueous solutions. The Sakaguchi

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reaction involving the decomposition of guanidino groups of the arginine residues, which is a complicated and time consuming process. Preliminary studies showed that the Lowry reagent (Lowry et al., 1951), Coomassie brilliant blue protein assays (Chan et al., 1995) and bicinchoninic acid protein assay (BCA) (Smith et al., 1985) gave only unsatisfactory responses to protamine concentrations ranging from 20 to 1000 μg/ml. Additionally, Bradford reagent with a sensitivity range of 100–1400 μg/ml protamine is also not sensitive enough for the quantification of protamine in low concentrated solutions (Bradford, 1976).

Fluorescence spectroscopy was introduced to improve the sensitivity limits for protamines. Fluorescence labelling of protamine with fluorescein (FITC) and tetramethyl-rhodamine (TRITC), respectively, was described before (Lochmann et al., 2004; Wehling et al., 1976). Disadvantages of these procedure are: (i) unpredictable side-reactions for the peptide during the purification after the labelling; (ii) free fluorescence dye can distort the quantification results; (iii) an expensive and time consuming procedure.

The derivatisation of amino acids with *ortho*-phthal-dialdehyde (OPA) was previously described in a pioneer work by Roth (Roth, 1971), further on, for high performance liquid chromatography and capillary electrophoresis applications (Albin et al., 1991; Chen et al., 1997, 2001; Kutlan et al., 2002; Lochmann et al., 1998; Molnar-Perl, 2001; Pi et al., 2000). Additionally, biogenic polyamines like spermine, spermidine, cadaverine and putrescine could be determined after derivating the primary amino groups of these molecules with OPA reagent (Conca et al., 2001; Kutlan et al., 2002; Venza et al., 2001).

The principal limitation of the OPA-derivatives of primary amino-groups (isoindols) is their instability. For this reason several studies have been carried out to characterise the stability of these molecules (Saito et al., 1994; Campins-Falcó et al., 2001; Moliner Martinez et al., 2004).

This report describes a new method to quantify protamine by derivating the primary amino groups with *ortho*-phthaldialdehyde (OPA) and 2-mercaptoethanol (ME), respectively, *N*-acetyl-L-cysteine (NAC) and their determination by fluorescence spectrometry. The measurements were carried out in a microtiter plate reader equipped with a pump unit for the derivatisation reagent, to develop a fast and easy quantification method for protamine and its salts.

## 2. Materials and methods

### 2.1. Materials

*Ortho*-phthaldialdehyde (OPA) pro analysis was obtained from Merck (Darmstadt, Germany). Protamine free base (PB), protamine sulphate (PS) and protamine chloride (PC) extracted from salmon sperm were purchased from Sigma (Steinheim, Germany). Unmodified oligonucleotides and phosphorothioate modified oligonucleotides (20 mer) were obtained from Biospring (Frankfurt, Germany). All other chemicals were of the highest available purity and were purchased from Sigma (Steinheim, Germany). Water (MQ-water) was purified with a Milli-Q Plus system obtained from Millipore (Schwalbach, Germany).

### 2.2. Methods

#### 2.2.1. OPA reagent

Solution A: 150 μM *ortho*-phthaldialdehyde (OPA) were dissolved in methanol. Solution B: 1.5 mM 2-mercaptoethanol (ME), respectively *N*-acetyl-L-cysteine (NAC) were dissolved in 10 ml borate buffer (0.1 M boric acid with potassium hydroxide adjusted to pH 9.4). The solutions A and B were mixed in a volume ratio 2:1 at 4 °C 2 h before use.

#### 2.2.2. Protamine sample preparation

Three milligrams of protamine free base (PB), protamine sulphate (PS) and protamine chloride (PC) were dissolved in highly purified, salt free water (MQ-water) under stirring at 25 °C. Afterwards, sample series of each protamine quality were prepared with a concentration of 50, 25, 10, 7.5, 5, 2, 1, 0.5, 0.25 μg/ml in borate buffer (0.1 M boric acid with potassium hydroxide adjusted to pH 9.4).

#### 2.2.3. Fluorescence spectra

The excitation- and emission-spectra of the isoindol-derivatives were determined with a luminescence spectrometer LS 50B Perkin Elmer (Überlingen, Germany). 500 μl of a 2 μg/ml PB solution

(in borate buffer pH 9.4) were incubated with 500 μl OPA/NAC reagent for 5 min and afterwards measured by scanning the excitation- and emission-spectra in 0.5 nm steps.

#### 2.2.4. Fluorescence measurements in microtiter plates

One hundred microliters of each protamine sample were transferred in a black 96-well microtiter plate obtained from NUNC (Wiesbaden, Germany). One hundred microliters of the OPA/ME or OPA/NAC reagent were automatically injected into each well. The measurement was performed at an excitation of $\lambda_{ex}$ 345 nm and at an emission of $\lambda_{em}$ 450 nm with a FLUOstar Galaxy microtiter plate reader (BMG-Labtechnologies, Offenburg, Germany). The FLUOstar temperature was set to 25 °C. The gain of the microtiter plate reader was fixed at 30 for the sample series of 0.25–10 μg/ml and for the sample series of 5–50 μg/ml at 79. The mean fluorescence intensity (MFI) was calculated as an average of seven independent measurements.

#### 2.2.5. BCA protein quantification method

The protamine free base content was measured with a commercially available bicinchoninic acid assay (BCA-assay by Uptima, France). One hundred microliters of protamine free base aqueous solutions (0.5, 5, 50, 500 μg/ml) were incubated in a 96-well microtiter plate with 200 μl BCA solution mixture following the manufactors manual. The adsorption was measured at $\lambda_{max}$ 550 nm. This method was used for cross-validation.

#### 2.2.6. Protamine-oligonucleotide nanoparticle preparation

Protamine-oligonucleotide particles were manufactured as described before (Gonzalez Ferreiro et al., 2001; Junghans et al., 2000, 2001). In detail, solutions of 15–150 μg/ml PB in water were prepared from a stock solution containing 2.5 mg/ml PB. To 1 ml of the diluted solution 30 μg of unmodified, respectively, phosphorothioate modified oligonucleotides were added and mixed for 5 s. After incubating for 2 h at room temperature particle preparations with different ratios of oligonucleotides/PB were centrifuged at 20,000 × *g*, 25 °C for 60 min. The supernatants were separated from the pellets. Five hundred microliters of a 20% (w/w) solution of polyphosphate in MQ-water were added to dissolve pellets and the samples were kept for 3 h in a thermocycler (1000 rpm, 60 °C). Each 100 μl of the solubilised pellet sample was measured as described for PB in Section 2.2.4.

## 3. Results and discussion

### 3.1. Reaction of OPA with primary amino groups in general

Based on the pioneering work of Roth (Roth, 1971), who used *ortho*-phthaldialdehyde (OPA) in combination with 2-mercaptoethanol to quantify amino acids, the reaction of protamine with OPA in the presence of various SH-group containing agents leads to isoindol derivatives of the primary amino groups. The reaction schema is shown in Fig. 1. The advantage of this reaction strategy is that the educts have no interference with the fluorescence spectra of the resulting isoindoles. Thus, no further separation procedure is necessary after derivatisation with the OPA/ME or OPA/NAC reagent. This is a major advantage in comparison to other labelling procedures like isothiocyanate or succinimidylester which have to be purified afterwards. However, the absence of a separation of educt and product was a precondition for the development of a one step microtiter plate quantification method.

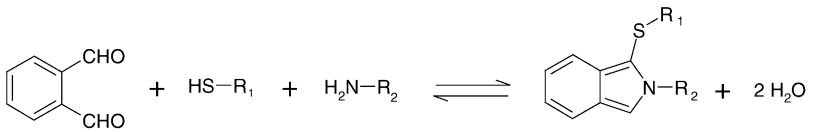


Fig. 1. Reaction pathway of *ortho*-phthaldialdehyde (OPA) with SH-group containing molecules ($R_1$; 2-mercaptoethanol (ME) or *N*-acetyl-L-cysteine (NAC)) and primary amino groups ($R_2$; protamine) resulted in a 1-alkylthio-2-alkyl-substituted isoindol (Albin et al., 1991; Mengerink et al., 2002).

The optimal molar ratio for both OPA reagents, ME and NAC, was found to be 1:50 (OPA/ME, NAC). A concentration of 100 μM OPA in the reagent was used and finally mixed 1:1 (v/v) with the sample. In our study the pH of the reaction could be optimised at 9.4 with a basic borate buffer, but also a phosphate buffer can be used at the same pH.

### 3.2. Fluorescence spectra of the protamine isoindol derivative

The excitation- and the emission-spectra of the isoindol derivative of protamine free base are shown in Fig. 2. The highest excitation ($\lambda_{ex}$) was determined at 345 nm and for the emission spectrum ($\lambda_{em}$) a maximum was measured at 450 nm. No difference between the isoindoles of OPA/ME and OPA/NAC was detectable (data not shown). These measurements are confirming the findings of previous studies dealing with amino acids and OPA reagents (Molnar-Perl, 2001; Molnar-Perl and Bozor, 1998).

### 3.3. Kinetics of derivatisation reactions

We started the development of a protamine analytic with the OPA/ME reagent, well known for the on-column derivatisation of amino acids in HPLC and CE (Albin et al., 1991; Lochmann et al., 1998). In general, it was found that the stability of the isoindoles with OPA/ME reagent was not high enough for a microtiter plate quantification method. A fast and non-reproducible decrease of the fluorescence signal was found (Fig. 3). This result confirmed the finding of other groups with OPA/ME reagent and amino acids (Molnar-Perl, 2001). In contrast, the stability of the isoindoles of the OPA/NAC reagent allows the manual application, besides the automatic application, of the OPA reagent on the microtiter plate. Thus, we applied to all following measurements the OPA/NAC reagent, which showed a sufficient stability of the isoindoles after 10 min for approximately 30 min (Fig. 3). After this time period, the fluorescence intensity slightly increases for 2 h before the signal intensity decreases (data not shown). The reaction time between OPA/NAC reagent and protamine

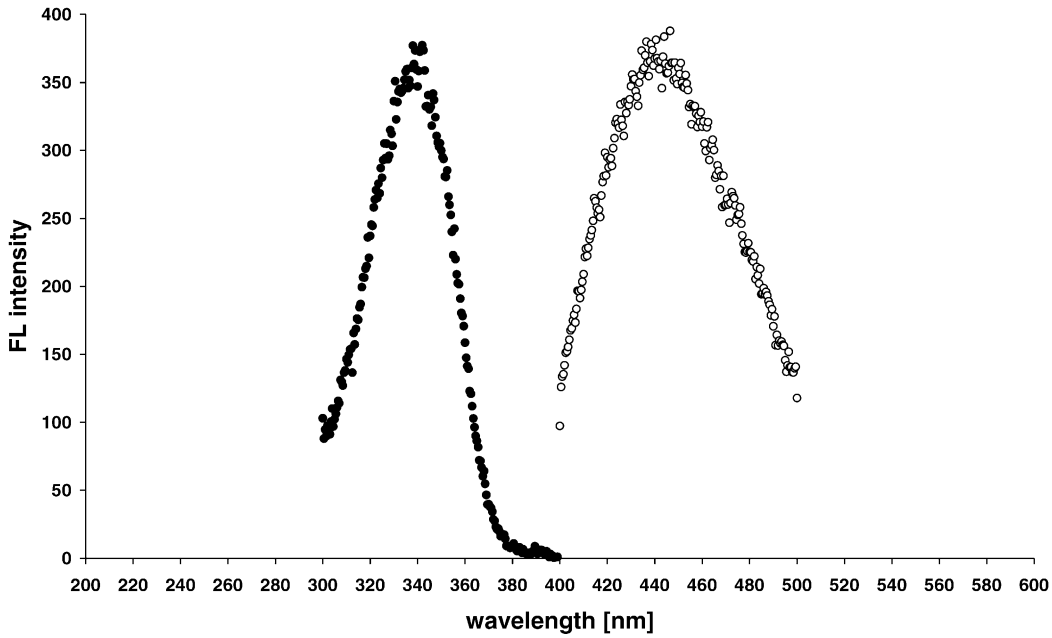


Fig. 2. Excitation- (●) and emission-spectra (○) of the protamine-isoindol-derivative (OPA/NAC reagent) with a maximum for $\lambda_{ex} = 345$ nm and $\lambda_{em} = 450$ nm.

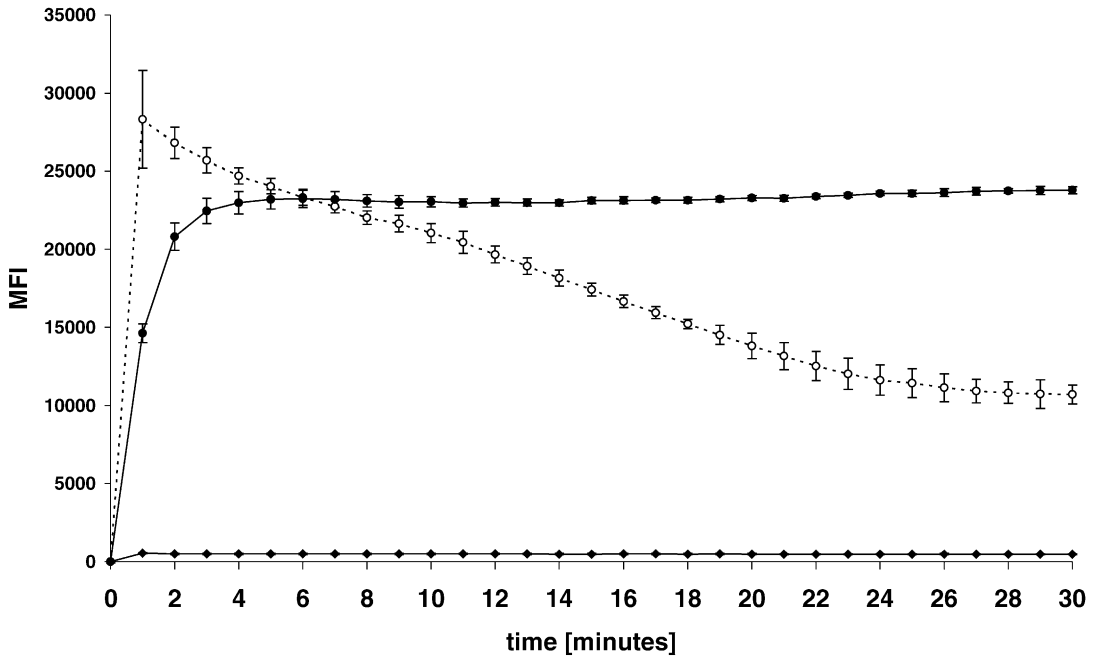


Fig. 3. Measurement of the kinetics of the fluorescence intensity (MFI). Reaction of 5 μg/ml protamine free base with OPA-ME reagent (○) and OPA-NAC reagent (●). Blank values with OPA-NAC reagent in borate buffer are visualised as (◆).

should be at least 7 min which is in correlation to earlier studies on the derivatisation of amino acids (Molnar-Perl, 2001; Molnar-Perl and Bozor, 1998).

### 3.4. Quality of the fluorescence signals

The quality parameters for the sample series of protamine free base, protamine chloride and protamine sulphate are given in Table 1. *F*-test was performed with the variances of the ($f_1$) 0.25 μg/ml and ($f_2$) 10 μg/ml measurements ($n = 6$) to prove the linearity of the three calibration graphs. For protamine free base ($f_2/f_1 = 234$), protamine chloride ($f_2/f_1 = 163$) and protamine sulphate ($f_2/f_1 = 157$) a highly significant difference ($P = 0.001$) of the variances was found.

The limit of detection (LOD) of all analytes were found to be less than 1.1 μg/ml (110 ng per well). Additionally, the limits of quantification (LOQ) were found to be less than 1.7 μg/ml (170 ng per well).

### 3.5. Cross-validation of the OPA/NAC method with the BCA protein quantification assay

The cross-validation between OPA/NAC and the BAC protein quantification assay (reference method) was performed at 50 μg/ml PB. An *F*-test was calculated with both variances ($f_{1BCA} = 43.29$; $f_{2OPA}$

Table 1
Analytical properties corresponding to the determination of protamine and its salts by using OPA/NAC derivatisation

| Analyte | $a \pm s_a$ | $b \pm s_b$ | $r^2$ | $s_{xo}$ | LOD (μg/ml) | LOQ (μg/ml) |
|---|---|---|---|---|---|---|
| Protamine free base | 4506 ± 125 | −586 ± 73 | 0.9994 | 0.148 | 0.935 | 1.397 |
| Protamine chloride | 3567 ± 35 | 964 ± 42 | 0.9995 | 0.136 | 0.861 | 1.286 |
| Protamine sulphate | 4208 ± 148 | −918 ± 85 | 0.9992 | 0.172 | 1.081 | 1.615 |

Quality parameters of the calibration graphs ($y = ax + b$) are given as $s_a$ (ordinate standard deviation), $s_b$ (slope standard deviation), $r^2$ (regression coefficient), $s_{xo}$ (standard deviation of the method), limit of detection (LOD) and limit of quantification (LOQ).

= 3.57) and a significant difference was obtained ($P \geq 0.05$). Afterwards, a *t*-test including the different variances resulted a $P < 0.05$ for the mean values of both methods (BCA: 51.21 ± 6.58 μg/ml; OPA: 50.08 ± 1.89 μg/ml). Concluding these results, the reference method confirmed the new OPA quantification method.

### 3.6. Quantification of protamine in presence of oligonucleotides

The analytical application of the method described in this paper was applied to the quantification of protamine in phosphorothioate antisense oligonucleotide (PTO) samples. For this purpose it became clear that it is absolutely necessary to perform blank measurements with the OPA/NAC reagent and the used buffer or analytic matrix. In general, the blank fluorescence is the main factor limiting the sensitivity of this method. Therefore, the OPA reagent was stored at least for 2 h at 4 °C to eliminate the self-fluorescence of impurities. These impurities, e.g. ammonium ions, react with OPA in the presence of SH-groups and can also form fluorescent isoindoles (Kang et al., 2003).

Further side reactions with aromatic primary amino groups contained in DNA showed a much lower reactivity with a slower increase of the fluorescence signal and a 12 times less MFI after 7 min, correlated to the same amount of protamine free base. Thus, it was possible to determine the content of protamine free base entrapped in antisense oligonucleotides (PTO) containing nanoparticles, which were prepared as described (Junghans et al., 2000, 2001; Lochmann et al., 2004, in press). After the particle assembly, the different nanoparticle preparations were centrifuged and the pellets were separated from the supernatant. Further on, the pellets were dissolved with a polyphosphate solution. Afterwards, the protamine content of these samples was measured with the microtiter plate reader using OPA/NAC reagent as described above. It was shown that the protamine free base in the nanoparticles could be determined by this method without further separation step (Table 2).

The current data confirmed earlier results obtained from nanoparticle containing tetramethylrhodamine labelled protamine at a low protamine concentration, which demonstrates the sensitivity of our new method. At high protamine concentrations the derivatisation with OPA/NAC reagent was found to be less sensitive, in comparison to tetramethylrhodamine labelled protamine, which could be attributed to a quenching effect. Measurement carried out in the presence of unmodified oligonucleotides resulted to similar data, which indicates that our new method can also be applied to DNA containing samples (data not shown).

Table 2
Quantification of protamine free base (PB) in nanoparticle preparations in the presence of phosphorothioate oligonucleotides (PTO)

| Used in the preparation | | Found in the nanoparticle pellet | |
|---|---|---|---|
| PTO (μg) | PB (μg) | PB (μg) | SD |
| 30 | 15 | 9.8 | ±0.9 |
| 30 | 30 | 13.8 | ±0.4 |
| 30 | 45 | 18.5 | ±0.1 |
| 30 | 60 | 21.9 | ±0.3 |
| 30 | 75 | 23.2 | ±0.3 |
| 30 | 90 | 27.2 | ±0.6 |
| 30 | 150 | 30.7 | ±0.1 |

Standard deviations (±SD) were calculated from five independent measurements.

## 4. Conclusion

A fast and reliable method to quantify protamine in pharmaceutical samples was developed. Our analytical technique requires neither preliminary extraction with an organic solvent nor long multi-step procedures. Protamine can be quantified in the presence of unmodified and phosphorothioate modified oligonucleotides. Protamine free base, protamine sulphate and protamine chloride can be determined with a limit of detection less than 1.1 μg/ml and a limit of quantification less than 1.7 μg/ml analyte.

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