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Titrimetric determination of Cremophor® EL in aqueous solutions and biofluids
Part 2: Ruggedness of the method with respect to biofluids☆

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Abstract

A titration method for Cremophor® EL, as a multicomponent mixture commonly used as non-ionic emulgent for manufacturing certain parenteralia, was developed for quantitative routine analysis in biofluids. A coated wire electrode is used as the end-point indicator in potentiometric titrations of Cremophor® EL with sodium tetraphenylborate. The method tolerates a broad pH range, addition of alkanols and components of drug formulations and is sufficiently rugged. Reliable results are obtained at 20°C. Disturbing ions from biofluid matrices can be masked or complexed by addition of formaldehyde, ethylenediaminetetraacetic acid and sodium fluoride. Sodium hydroxide is used for the required adjustment of the samples to pH 10. Cremophor® EL spiked urine samples can be determined directly, whereas the true value of the emulgent content in the case of Cremophor® EL spiked plasma samples is achieved by means of a conventional method. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Cremophor® EL; Potentiometry; NIO-tensid-electrode; Biofluids

1. Introduction

Cremophor® EL (α-hydro-ω-hexadecyloxypoly-(oxyethylene)) obtained from 1 mol castor oil and 35 mol ethylene oxide as a multicomponent mixture [1] is a commonly used non-ionic emulgent and solubiliser for the preparation of aqueous solutions of hydrophobic substances. Its components are given in Fig. 1. Even though Cremophor® EL (CrEL) is used for manufacturing parenteralia since it does not cause haemolysis [2], various other side-effects such as anaphylactoid [3,4] and anaphylactic reactions [5,6], respectively, changes in plasma lipoptoteins [7] and plasma

☆ Dedicated to Professor Siegfried Ebel on the occasion of the 65th anniversary of his birthday.

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viscosity [8], hemodynamic effects [9], nephrotoxicity [10], neurotoxicity [11] and inhibition of the protein kinase C [12,13] demand pharmacokinetic investigations of the excipient.

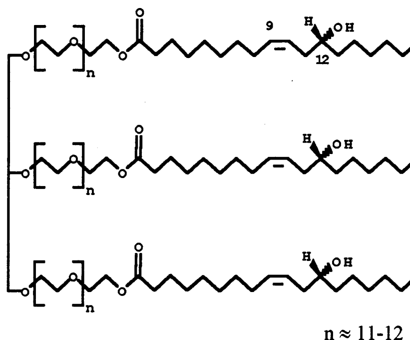
Despite extensive clinical use of CrEL containing parenteral dosage forms and despite the well-known and chemotherapy-relevant CrEL induced reversal of multidrug resistance (MDR) [14] the pharmacokinetics of the emulgent have yet to be described precisely.

Up to date only a few investigations on the kinetics of CrEL have been carried out.

Neumann [15] investigated the elimination of ^{14}C-marked CrEL from female Sprague–Dawley rats. After intravenous application of 50 mg ^{14}C-CrEL/kg body weight, 60% of the radioactivity was excreted in the urine and 40% in the faeces, which indicates a biliary secernment. Elimination occurred rapidly: 91% of the radioactivity, excreted in the urine, was found in 24 h and 75% of the radioactivity, excreted in the faeces, was also found also within 24 h. Extremely small amounts of ^{14}C-CrEL were observed in breath. Not more than 1.5% of the total radioactivity remained in the body of the rat 6 days after application with a recovery rate of 100%. Since this experiment is not transferable to humans, Webster et al. [16] developed a bioassay for the determination of CrEL, suitable for pharmacokinetic studies in patients. The assay is based on the above mentioned MDR, a mechanism of cellular resistance to chemotherapy, in which tumor cells express elevated levels of membrane transport proteins, e.g. P-glycoproteins, that actively pump a broad spectrum of structurally unrelated drugs out of the cell. CrEL is able to block this mechanism responsible for the MDR. The extent of the inhibition of this pump is dose-dependent and leads to a reverse of the MDR at high concentrations. Flow cytometry was used to measure the increase of intracellular daunorubicin levels at equilibrium in multidrug-resistant human T-cell leukemia cells (CEM/VLB_{100}) in the presence of a series of different CrEL concentrations. The purpose of the study was to determine whether the CrEL plasma levels achieved during paclitaxel (which is also solubilised by CrEL) therapy are sufficient to inhibit the activity of the P-glycoprotein or not.

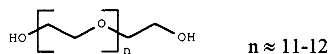
Hydrophobic portion:

1) Esters of ricinoleic acid and glycerol polyglycol ethers
2) Esters of ricinoleic acid and polyglycols
3) Esters of ricinoleic acid the secondary hydroxyl groups of which are oxethylated to a minor extent
4) Diesters and higher esters of polyglycols with ricinoleic acid the secondary hydroxyl groups of which are additionally esterified with ricinoleic acid
5) Esters of oleic acid and polyglycol
6) Unreacted castor oil



Hydrophilic portion:

1) Glycerol polyglycol ethers
2) Polyglycols



n ≈ 11-12

Fig. 1. Components of CrEL [1].

Webster et al. proved that under the chosen therapy conditions CrEL plasma levels essential to reverse MDR are reached.

In 1996 this bioassay was used for another determination of CrEL [17] in plasma samples of patients undergoing a modified treatment with paclitaxel. The bioassay provided worthy data concerning the reverse of MDR, but it only captured the unknown 'MDR active' components. No statements could be made on the elimination of CrEL from the human organism.

Sparreboom et al. [18] established an RP-HPLC method with sample preparation after saponification of CrEL in alcoholic KOH, followed by extraction of the released ricinoleic acid with chloroform and subsequent derivatisation with 1-naphthylamine. The resulting products were separated on an analytical column packed with Spherisorb ODS-1 material and a methanol-acetonitril-potassium phosphate buffer mobile phase with detection at 280 nm. Pharmacokinetic studies of CrEL in mice and patients receiving CrEL solubilised paclitaxel have demonstrated the applicability of this assay, which required only microvolumes (20 μl) of plasma. However, only the ricinoleic acid containing components of the emulgent can be monitored, since all other fatty acids are not to be differentiated from the endogenous fatty acids.

Sparreboom et al. [19] developed also a colorimetric dye-binding microassay for the quantification of the emulgent. The procedure is based on rapid binding of Coomassie brilliant blue G-250 to CrEL. The binding of the dye to CrEL causes a shift in the absorption maximum, which is monitored by a microplate absorbance reader. Sample preparation includes plasma protein precipitation with acetonitril and extraction with *n*-butylchloride, which allows again merely the investigation of the hydrophobic portion of the emulgent.

Indeed, up to date there is no satisfactory method available for the quantitative analysis of all CrEL components. In order to investigate the pharmacokinetics of CrEL in detail an adequate titrimetric method was developed. Optimisation and validation of the potentiometric titration of pure aqueous sample solutions have been described in part 1 [20]. Herein, the applicability of the method with respect to biofluids and its ruggedness will be discussed.

2. Materials and methods

2.1. Chemicals

CrEL was a gift from BASF (Ludwigshafen, Germany). Barium chloride of suprapur grade was obtained from Merck (Darmstadt, Germany). Sodium tetraphenylborate (Na-TPB) of analytical grade was purchased from Acros (New Jersey, USA). HPLC grade methanol was purchased from Janssen Chimica (Geel, Belgium). The intravenous preparation of Sandimmun® was obtained from Novartis Pharma (Nürnberg, Germany).

Preparation of titrant, $BaCl_2$-containing activator, CrEL stock and calibration solutions as well as CrEL dilutions is described in part 1 [20].

2.2. CrEL spiked biofluids

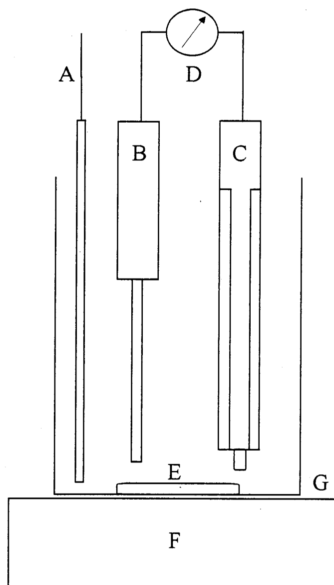
Whole blood, plasma, serum and urine were obtained from the authors or from healthy volunteers. If not stated otherwise the CrEL-spiked biofluids were prepared as follows.

An aliquot of the required CrEL dilution was pipetted into a 50-ml beaker, followed by either 1 ml of plasma or serum or 10 ml of urine, respectively. After addition of 10 ml activator the sample was diluted with aqua dest. to 40 ml. Then, solution was stirred for 1 min before starting the titration.

2.3. Anti-interference solution and CrEL spiked biofluids

The in situ prepared anti-interference solution (AIS) consists of the solutions described in Sections 2.3.1, 2.3.2, 2.3.3 and 2.3.4, which were exclusively prepared with freshly boiled and carbonate-free water. These solutions are used for sample preparation as follows.

An aliquot of the required CrEL dilution was pipetted into a 50-ml beaker, followed by either 1 ml of plasma or serum or 10 ml of urine, respec-



A: titrant supplying
(Titrino 702 SM with 5 mL-dosage piston)

B: NIO-tensid-electrode 6.0507.010
(Metrohm AG, Herisau, Switzerland)

C: Ag/AgCl-reference-electrode 6.0726.100
inner electrolyte: 3 M KCl-solution
in-between electrolyte: 1 M NaCl-solution
(Metrohm AG, Herisau, Switzerland)

D: potentiometer
(Titrino 702 SM, Metrohm AG, Herisau, Switzerland)

E: stirring fish

F: magnetic stirrer E549
(Metrohm AG, Herisau, Switzerland)

G: 50 mL beaker

Fig. 2. Titration apparatus.

tively. To the sample adjusted to pH 10 with 1 M sodium hydroxide solution were pipetted 1 ml each of formaldehyde, EDTA and sodium fluoride solutions, if not stated otherwise. After addition of 10 ml activator the sample was diluted to 40 ml with aqua dest. and stirred for 1 min before starting the titration.

2.3.1. Sodium hydroxide solution

A 1-M sodium hydroxide solution was prepared by dissolving the required amount of sodium hydroxide (suprapur grade; obtained from Merck, Darmstadt, Germany) in aqua dest.

2.3.2. Formaldehyde solution

The 35% (w/v) aqueous formaldehyde solution was of suprapur grade (obtained from Merck, Darmstadt, Germany).

2.3.3. Sodium fluoride solution

A 0.1-M sodium fluoride solution was prepared by dissolving the required amount of sodium fluoride (suprapur grade; obtained from Merck, Darmstadt, Germany) in aqua dest.

2.3.4. Ethylenediaminetetraacetic acid solution

A 0.1-M ethylenediaminetetraacetic acid (EDTA) solution was prepared by dissolving the required amount of EDTA disodium salt dihydrate (suprapur grade; obtained from Merck, Darmstadt, Germany) in aqua dest.

2.4. Titration apparatus

Fig. 2 shows a sketch of the employed titration apparatus. Titration parameters are summarised in Table 1. Storage, conditioning and cleaning of the non-ionic (NIO)-tensid-electrode are described in part 1 [20]. Titration curves were evaluated with TiNET 2.1 software (Metrohm, Herisau, Switzerland).

Table 1
Optimised standard titration conditions, if not stated otherwise

Regulation of titration	Monotone equivalent point titration
Indication mode	Potentiometry
Temperature	Ambient
Start delay	30 s
Titrant increment	0.2 ml
Flow rate of increment	1 ml/min
Drift	5 mV/min
Waiting period	360 s
EP criterion	15 mV
Titrant	0.002 M NaTPB solution
Activator	0.1 M $BaCl_2$ solution

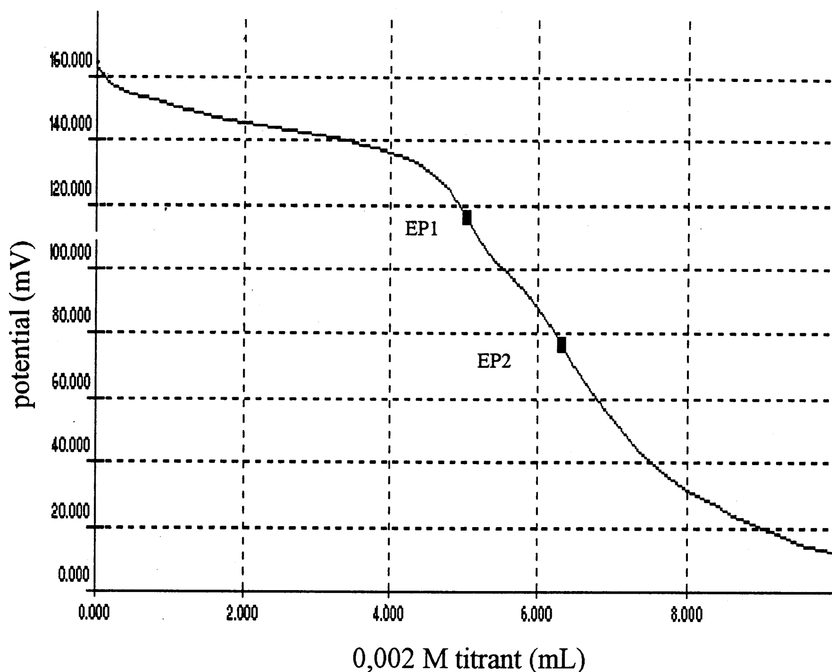


Fig. 3. Typical titration curve of CrEL.

3. Results and discussion

Non-ionic surfactants like CrEL are characterised by their oxyethylene side chains, which are able to form complexes with a broad spectrum of mono- and divalent cations [21], so-called pseudocations, which are stabilised by ion-dipole interactions. Their conformation is similar to that of crown ether complexes. The complex forming process is called activation. Non-ionic surfactants in their activated form can be titrated with an adequately large anion, e.g. the tetraphenyllborate (TPB) anion, which forms nearly water-insoluble ternary complexes with pseudocations in aqueous solutions. The end-point of this potentiometric titration is indicated by means of a TPB-sensitive coated wire electrode, which reacts to a surplus of TPB ions. The optimised and validated method has been described in part 1 [20]. This method is able to determine all components of CrEL except unreacted castor oil, which is present at less than 3%, and is therefore a promising tool for investigating the pharmacokinetics of CrEL. The titration curve (Fig. 3) is characterised by two points of inflection (EP1; EP2). EP2 is more pronounced and therefore used for construction of the calibration straight line (Fig. 4).

Following experiments are based on the described procedure.

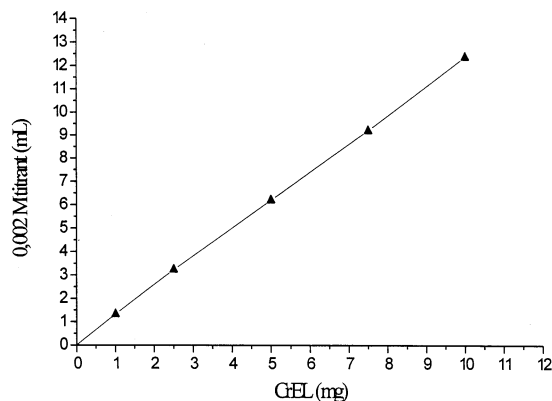


Fig. 4. Calibration straight line constructed from EP2.

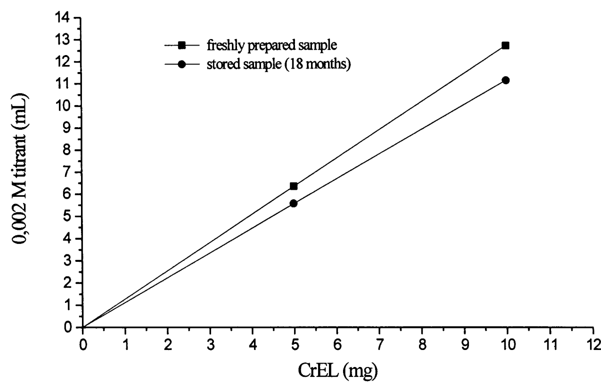


Fig. 5. Calibration straight line influenced by sample age.

3.1. Ruggedness

3.1.1. General parameters

Parameters, which were supposed to influence the titration results, were tested and optimised as follows. As mentioned in part 1 [20] samples containing 5 mg CrEL were employed since minimum standard deviations (S.D.) of the titration results are obtained in this mass range. Under each described condition three titrations were performed and the results were averaged.

3.1.1.1. Temperature. Although the Nernst equation is not valid because of the surface activity, the substantivity as well as the micelle building capability of CrEL [20], the temperature plays an important role during titration, since, for example, the potential difference measured between the NIO-tensid-electrode and the reference electrode is directly temperature dependent. The titration method was tested at 0, 20 and 40°C, temperatures well tolerated by the NIO-tensid-electrode. It was observed that with increasing temperature consumption of the 0.002 M titrant decreased and EP1 (which is not used for analysis [20]) becomes less pronounced. Reliable results were obtained at 20°C, which was used for all following experiments.

3.1.1.2. pH value. With respect to acidosis and alkalosis, pH values of biofluids like plasma [22] and urine [23] are in the range of 4.8 to 7.8, which is covered by the NIO-tensid-electrode with a recommended pH range between 3 and 9 for titration [21]. Test titrations at pH values of 3.4, 5.4, 7.4, 9.4 and 11.8 confirmed the titration results to be virtually independent of the adjusted pH value in the tested range. However, carbonate free solutions are required for alkaline samples which avoid precipitation of $BaCO_3$ clinging to the electrodes and falsifying the titration results.

3.1.1.3. Alkanols. Alkanols are used to reduce disturbing foam formation and surface activity of emulgents. Addition of a few drops of methanol to samples does not influence the titration result as discussed in part 1 [20].

3.1.1.4. Sample age. As observed during capillary electrophoresis analysis aqueous solutions of CrEL are not stable [24]. Therefore freshly prepared samples were investigated in comparison to samples stored for 18 months at ambient temperature. Fig. 5 shows that the stored samples lead to lower titration results in comparison to the freshly prepared ones. Possibly the hydrolysis of CrEL components into low molecular polyglycol derivatives causes the decrease of titrant consumption. Hence, only freshly prepared aqueous samples should be analysed.

3.1.1.5. Freezing/thawing. Freshly prepared samples and samples subjected to three freeze-thaw cycles (12 h freezing and 12 h thawing each) were analysed. It could be shown that freeze-thaw cycles do not influence the titration results. Hence, samples can be stored for a short time by freezing.

3.1.1.6. Drug formulation. Clinically used intravenous preparation of Sandimmun® contains the drug cyclosporin A and CrEL and ethanol as excipients. To study whether samples containing this drug formulation are titratable or not, they were adjusted to the content of 5 mg CrEL and submitted to the standard procedure. Titration results and calibration line corresponded. Influences from components of the drug formulation on the titration can therefore be neglected.

3.1.1.7. Co-medication. Some surface-active drugs (e.g. cetylpyridinium chloride and benzalkonium

chloride) and some drugs, which are able to form ions of a defined size (e.g. ambroxol, clotrimazol, and chloroquine) interfere with the ion-sensitive membrane of the electrode or lead to precipitation with the TPB anion [25]. Other co-medications do not disturb the titration method.

3.2. Titration of biofluids

Quantitative CrEL determinations in the context of pharmacokinetic studies in patients, treated with CrEL containing parenteralia, can only be carried out, if the transferability of the optimised and validated titration method to biofluids is established. The titration of CrEL in biofluids such as urine, plasma and serum might be influenced by numerous inorganic and organic components (Table 2). There are two possibilities to overcome the interferences: development of a suitable sample preparation technique or of a direct titration method.

Standard sample preparation procedures such as liquid/liquid extraction and solid phase extraction are unsuitable for CrEL because of the surface active characteristics of the solubiliser. Since non-ionic surfactants like CrEL interact with most of the exchange resins [26] disturbing cations cannot be eliminated by this measure.

A CrEL-polyacrylic acid-complex can be precipitated and isolated with polyacrylic acid in acidic milieu at increased temperature. However, these precipitations occur only at high CrEL levels [27] and are therefore unsuitable for the determination of small amounts of emulgent in biofluids.

Another alternative is the adsorption of CrEL to high disperse silicon dioxide (Aerosil®) in organic solvents, which can be separated successively. However, after transfer into aqueous solutions adsorption phenomena of the emulsifying agent on the surface of Aerosil® are maintained, leading to incorrect results [28–32]. Consequently, this sample preparation procedure is also ruled out in favour of the direct titration of biofluids, which remains the method of choice. For that purpose addition of AIS with selective masking or precipitating reagents (Table 2) has been tested for routine analysis of biofluids, spiked with 0.2, 2.5, 5.0, 7.5 and 10 mg CrEL, respectively, titrated apart from blank samples three times each. The titration solutions contained either 10 ml urine or 1 ml plasma or 1 ml serum.

3.2.1. Urine

A complete activation of all CrEL components in spiked urine with regular amounts of activator is prevented by precipitation of barium salts of

Table 2
Interfering components of biofluids

Interfering components of		Interfering mechanism	Measures against interferences	
Urine	Plasma		Component of AIS	Anti-interference mechanism
K^+		Water-insoluble complexes with TPB-ions	NaOH	No precipitation at pH ≥ 7
NH_4^+		Water-insoluble complexes with TPB-ions	CH_2O	Hexamethylene-tetramine formation
Bivalent heavy metal ions (e.g. Cu^{2+})		Water-insoluble complexes with TPB-ions	EDTA	Complexation
Trivalent heavy metal ions (e.g. Fe^{3+})		Water-insoluble complexes with TPB-ions	NaF	Complexation
SO_4^{2-}		Water-insoluble salts with Ba^{2+}	–	Ba^{2+} surplus
CO_3^{2-}		Water-insoluble salts with Ba^{2+}		Ba^{2+} surplus
–	Proteins	Emulgent binds to surfaces	–	–

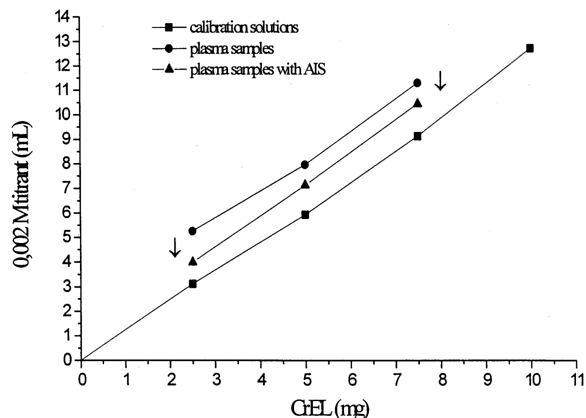


Fig. 6. Calibration straight line of plasma samples influenced by AIS.

native urine constituents. Therefore a sufficient surplus of activator (5 ml) was added to the urine samples. In contrast, no disturbing precipitation was observed after addition of the titrant to native urine.

Regular titration curves with well defined points of inflection were obtained for 0.2, 2.5, 5.0 and 7.5 mg CrEL spiked urine samples, respectively. The resulting straight line of the titration results and the calibration straight line corresponded well. Urine samples with 10 mg CrEL or more provide irregular titration curves and therefore imprecise results.

These preliminary experiments showed that urine samples containing CrEL in the range from 0.2 to 7.5 mg can be titrated directly with a surplus of activator.

3.2.2. Plasma

Even though CrEL spiked plasma samples did not lead to any precipitation either with the activator or with the titrant, direct titration of spiked plasma samples is not as simple as for spiked urine samples. Reliable results were only obtained for plasma samples containing 2.5, 5.0 and 7.5 mg CrEL, respectively. As shown in Fig. 6 the straight line obtained from the titration results does not fit with the calibration straight line, indicating that plasma components lead to an increased consumption of titrant. Therefore interfering plasma components have to be eliminated for accurate titration results.

3.2.3. Serum

In comparison to spiked plasma samples the CrEL spiked serum samples showed no differences in the titration results. Since the emulgent CrEL might be adsorbed to the surface of the coprecipitated fibrin during preparation of serum samples, further experiments were carried out exclusively with plasma.

Summarising the preliminary results with CrEL spiked biofluids a direct titration of spiked urine samples seems possible upon addition of a surplus of activator. In contrast, pretreatment of spiked plasma samples is necessary in order to overcome inherent interferences.

3.3. Measures against interferences

3.3.1. Composition of the AIS

The choice of suitable reagents for the AIS is governed by the type of interfering components in biofluids such as potassium, ammonium and heavy metal ions [22,23].

Since potassium ions are usually identified by Kalignost® (Na-TPB), sodium hydroxide solution was added to the samples to avoid K-TPB precipitation in the neutral or acidic milieu of the biofluids.

Ammonium ions were eliminated either by adding formaldehyde to form hexamethylenetetramine or by adjustment of the pH value to 10.

Complexation of heavy metal ions (except barium) is also necessary, since they would precipitate with TPB ions. In addition heavy metal ions might form less stable pseudocations with CrEL components [33], leading to incorrect titration results.

Fluoride as complex ligand has the advantageous side-effect of a potent inhibition of esterases, avoiding enzymatic hydrolysis of CrEL components.

Even if EDTA and fluoride are known to form complexes with barium ions of the activator, no disturbances could be observed under the given, carefully selected conditions.

A complete elimination of the interfering ions contained in biofluids is managed with the combination of those reagents in sufficient amounts [23]. In detail it is warranted by adjustment of the

samples to pH 10 by 1 M NaOH and by addition of 3 ml AIS consisting of 1 ml of a 35% formaldehyde solution, 1 ml of a 0.1-M EDTA solution and 1 ml of a 0.1-M NaF solution per 40 ml titration solution. Optimisation of the composition of the AIS is described in Section 3.3.4.3.

The rather complicated relations within the titration solutions containing biofluids made theoretical considerations unclear. As a consequence it was tested whether CrEL spiked biofluids with a sufficient volume of AIS and a surplus of activator solution provide satisfactory titration results.

3.3.2. Testing of AIS in aqueous CrEL samples

In a first step the influence of AIS on the titration of aqueous CrEL-containing samples was tested. Since the titration results corresponded well with the calibration straight line AIS does not disturb the titration and can be employed as additive for sample preparation.

3.3.3. Determination of the true value of the CrEL content in urine samples

3.3.3.1. Testing of AIS in CrEL spiked urine samples. In preliminary tests CrEL spiked urine samples could be titrated without any problems with a surplus of activator, yet in view of the extremely varying compositions of urine AIS has been added to the samples.

The titration results of those CrEL and AIS containing urine samples resulted in a straight line, which corresponded with the CrEL calibration straight line with appropriate S.D. in the same order of magnitude [20]. Thus AIS can be employed without limitation as additive for the 'anti-interference' of ions in urine samples.

3.3.3.2. Ruggedness with respect to strongly varying urine compositions. A major problem of the analysis of biofluids is the strongly inter- and intraindividual variation of urine compositions. Corresponding effects on the titration were tested with AIS containing urine samples, spiked with 2.5, 5.0, 7.5 mg CrEL.

To test the influence of interindividual urine compositions on the titration results, CrEL spiked urine samples of two male and two female test persons were examined. The resulting straight lines of the titration results and the CrEL calibration straight line corresponded in each case. Thus interindividual differences in the urine composition do not influence the titration results.

To test the influence of different intraindividual urine constituent concentrations on the titration results urine was restricted i. vac. to a fourth of its initial volume and then spiked with CrEL. The resulting straight line of the titration results and the CrEL calibration straight line again corresponded well. Thus concentrated urine likewise does not influence the titration.

High potassium ions concentrations could become particularly critical for the applied titration method. With physiological K^+ elimination from 1.4 to 3.1 g in 500–2000 ml urine/24 h, maximum K^+ concentrations amount up 60 mg/10 ml.

In order to study the influence of K^+ concentrations, pure aqueous CrEL samples were adjusted with KCl to the simple, double and threefold values of the estimated maximum K^+ concentration. Again all resulting straight lines of the titration results and the CrEL calibration straight line corresponded. Under the selected conditions even unphysiologically high K^+ concentrations do not disturb the titration.

Thus upon addition of sufficient volumes of activator and AIS to the samples the true value of the CrEL content in urine can be determined reliably by means of the described titration method. For urine samples intra- and interday standard deviations ($S.D._{intra}$, $S.D._{inter}$) at three control levels are listed in Table 3.

3.3.4. Determination of the true value of the CrEL content in plasma samples

3.3.4.1. Testing of AIS in CrEL spiked plasma samples. Fig. 6 shows the influence of AIS on the titration results of CrEL spiked plasma samples. The resulting straight line of the titration results is parallel both to the CrEL calibration straight line and that of the CrEL spiked plasma samples without AIS and located nearly equidistantly between them. In the case of the 7.5-mg CrEL and AIS containing plasma sample a deviation of only 15% from the true value of the content is ob-

Table 3
$S.D._{intra}$ and $S.D._{inter}$ for urine and plasma samples[a]

CrEL content (mg) of sample	Urine		Plasma	
	$S.D._{intra}$	$S.D._{inter}$	$S.D._{intra}$	$S.D._{inter}$
2.5	0.124	0.281	0.168	0.321
5.0	0.135	0.178	0.103	0.219
7.5	0.155	0.151	0.174	0.213

[a] Calculated from six observations for each control level.

served, which is tolerated in bioanalytics. This is a clear and sufficient improvement of the method compared to a 7.5-mg CrEL spiked sample without AIS, which showed a deviation of ~24%. Nevertheless an undisturbed titration is not achieved. Therefore the resulting straight lines are used as calibration straight lines for the precise determination of CrEL contents in plasma samples. Therewith, the S.D. of the titration results for the plasma samples both with and without AIS were determined to ~0.05 for the upper and to ~0.18 for the lower concentration range. For plasma samples the $S.D._{intra}$ and $S.D._{inter}$ at three control levels are listed in Table 3.

The limit of determination for CrEL spiked plasma samples could be lowered slightly by means of AIS from 2.5 to 2.0 mg in comparison to samples without AIS. Hence this titration method is suited for pharmakokinetic investigation of CrEL in patients receiving clinical formulation of the antineoplastic agent paclitaxel as a 3-h infusion at dose levels ranging from 100 to 225 mg/m^2 (corresponding to CrEL doses of 8.3–18.8 ml/m^2) with CrEL plasma concentration levels declining from ~7.5 mg/ml to 2.5 mg/ml within 24 h [34].

In comparison, immunosuppressive therapy with i.v. formulation of cyclosporin leads finally to CrEL plasma concentrations in patients which are below the limit of quantitation (LOQ). This obstacle is overcome with enlarged volumes of plasma allowing pharmakokinetic investigations of CrEL also at these plasma levels. The influence of the plasma concentration on the titration results is discussed in Section 3.3.4.2.

3.3.4.2. Influence of the plasma concentration on the titration result. Samples containing 5 mg CrEL, 1, 2 and 3 ml plasma, respectively, and AIS were titrated in order to examine the influence of different plasma amounts on the titration results. Fig. 7 shows a linear dependence between the consumption of the titrant and the added plasma amount.

Probably plasma contains a certain amount of interfering substances, which are not eliminated by AIS and therefore cotitrated with CrEL. Surprisingly plasma samples without addition of CrEL do not show any EP in control titrations.

3.3.4.3. Optimisation of AIS. Since a closer approach of the resulting straight line of CrEL spiked plasma samples towards the CrEL calibration straight line was striven for it has been tested whether a variation of the concentrations of the individual components of the AIS could improve the titration conditions or not. For this purpose

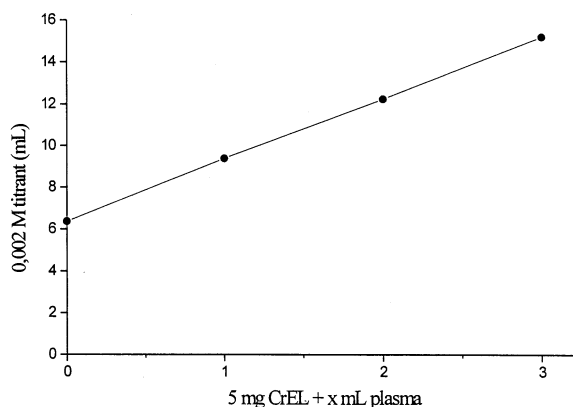


Fig. 7. Influence of plasma concentration on titration results.

AIS of different compositions were added to a base titration solution (BTS) containing 5 mg CrEL, 1 ml plasma and 10 ml activator. The resulting straight lines of the titration results were compared both to the resulting straight line of the genuine BTS and to the CrEL calibration straight line.

Addition of 1, 2 and 3 ml of the 0.1-M NaF solution, respectively, to the BTS led independently of the added volumes to a non-significant approach of the resulting straight lines to the CrEL calibration straight line. However, the adjustment of the BTS to pH 10 was advantageous for the complexing effect of EDTA and the masking effect of formaldehyde. Additionally to the pH effect a further approach of the titration resulting straight line to the CrEL calibration straight line was observed with the addition of 1 ml of the 0.1-M EDTA solution. However, this improvement is cancelled by further addition of 1 or 2 ml of the EDTA solution to the BTS, which therefore has to be strictly avoided.

The addition of just 1 ml of the 35% formaldehyde solution had a positive influence on the titration results, which could not be further improved.

This also applies for the initial composition of AIS, which could not be optimised with respect to all components.

3.3.4.4. Conventional method for CrEL determination in plasma. Summarising the results of the preceding investigations for the determination of the true value of the CrEL content in plasma the following methodology evolves as a conventional method: 2.5 and 5 mg CrEL containing plasma samples are used as calibration solutions in order to create a two-point calibration within the range of content with the smallest S.D., since the plasma concentration dependent calibration straight line does not cross the origin of the Cartesian coordinates. The calibration solutions are adjusted with volumes of plasma, the content of which may be expected in context with pharmakokinetic studies. Thus, the CrEL content of plasma samples of patients can be determined over the working range within bioanalytical accuracy by means of the resulting calibration straight line.

4. Conclusions

The titration of CrEL in biofluids with a $BaCl_2$ solution as activator and a TPB solution as titrant is disturbed by physiologically occurring ions, e.g. potassium, ammonium, heavy metal, sulfate and carbonate ions, since they form water-insoluble complexes or salts with the mentioned solutions. Interfering ions can be masked or complexed effectively at pH 10 with distinct amounts of formaldehyde, EDTA and NaF in the biofluid containing samples. This measure allows the determination of the true value of the CrEL content in urine samples. Since a direct approach to the true value of the CrEL content in the case of plasma samples was not successful, an accurate determination of the CrEL content in plasma samples requires the development of a conventional method referring to the plasma concentration.

The methods proved to be rugged and can be used for routine CrEL quantification in biofluids.

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