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Determination of Cremophor® EL in plasma after sample preparation with solid phase extraction and plasma protein precipitation

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Dedicated to Professor Dr. G. Blaschke on the occasion of the 65th anniversary of his birthday

Abstract

The non-ionic emulsifier Cremophor® EL can be quantified using a special potentiometric titration technique with barium chloride activation and precipitation with sodium tetraphenylborate. The end point of the titration is indicated by an ionsensitive coated wire electrode which responds to an excess of tetraphenylborate ions. Sample preparation is necessary to quantify the excipient in plasma of patients receiving ciclosporin formulations with Cremophor® EL (Sandimmun®), since plasma proteins cause disturbances of the titration. Solid phase extraction was tested with various sorbent materials. Although some of the sorbents yielded good extraction rates of Cremophor® EL from aqueous solutions, the extraction rates from plasma were significantly lower. Therefore, plasma protein precipitation with acetonitrile has been examined as an alternative to SPE and has been proved the superior method. Using the precipitation technique, a recovery rate of above 90% was achieved. Furthermore, the limit of detection from plasma was found to be 30 μg, in analogy to the determination from aqueous solutions. The combination of the plasma protein precipitation with the potentiometric titration allows quantitation and thus pharmakokinetic investigations of Cremophor® EL in patients treated with Sandimmun® after kidney-transplantation.

Keywords: Cremophor® EL; Non-ionic ethoxylate surfactant; Sandimmun®; Titrimetric determination of the excipient in plasma; Solid phase extraction; Plasma protein precipitation

1. Introduction

Cremophor® EL (CrEL) obtained from 1 mol castor oil and 35 mol ethylene oxide is a heteroge-

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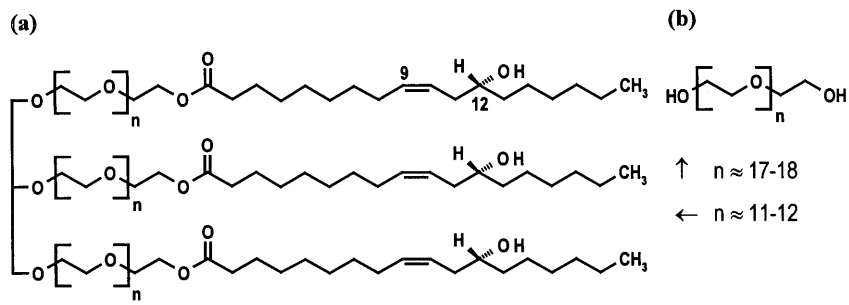
neous non-ionic emulsifier [1]. Its components are given in Fig. 1. Since it does not cause haemolysis, it is used for the manufacture of aqueous preparations for parenteral application [2] containing hydrophobic substances like ciclosporin (Sandimmun®) or paclitaxel (Taxol®). However, after parenteral application of CrEL diverse, partly serious side effects as anaphylactic [3,4] and anaphylactoid reactions [5,6], nephrotoxicity [7], neurotoxicity [8], changes on plasma lipoproteins [9] and plasma viscosity [10], haemodynamic effects [11] and inhibition of the proteinkinase C [12,13] are observed. These observations necessitate pharmakokinetic investigations of this excipient and thus methods for the quantitative analysis.

Although CrEL containing parenteral dosage forms are extensively used, the pharmakokinetics of the emulgent has not been described precisely yet.

Up to date only a few investigations on the kinetics of CrEL have been performed. The elimination of ^{14}C-marked CrEL from female Sprague–Dawley rats has been investigated by Neumann [14]. After intravenous application of 50 mg CrEL per kg body weight, 60% of the radioactivity was excreted with urine, 40% with faeces and extremely small amounts with breath. 91% of the radioactivity, excreted with urine, and 75% of the radioactivity, excreted with faeces, were eliminated within 24 h. Only 1.5% of the injected dose were observed in the body of the rat



Hydrophobic portion

1) Esters of ricinoleic acid and glycerol polyoxyethylene ethers (**a**)

2) Esters of ricinoleic acid and polyethylene glycol

3) Esters of ricinoleic acid with their secondary hydroxyl groups ethoxylated to a minor extent

4) Di- and higher esters of polyethylene glycol with ricinoleic acid with its secondary hydroxyl groups additionally esterfied with ricinoleic acid

5) Esters of oleic acid and polyethylene glycol

6) Unreacted castor oil

Hydrophilic portion

1) Glycerol polyoxyethylene ethers

2) Polyethylene glycol (**b**)

Fig. 1. Components of CrEL.

6 days after application. The recovery rate of the experiment was found to be 100%. Since this experiment is not transferable to humans, other methods had to be established to carry out pharmakokinetic studies in patients. Webster et al. [15,16] developed a bioassay for the quantitation of CrEL, which is based on the facility of CrEL to suppress multi drug resistance (MDR) induced by some cytostatic drugs [17]. After incubation of multidrug resistant human T-cell leukemia cells (CEM/VLB_{100}) with daunorubicin for 1 h reaching intracellular drug level equilibrium, the cells are mixed with CrEL containing plasma of patients treated with Taxol®. The intracellular daunorubicin levels are then measured by flow cytometry. With this assay, it was shown that plasma levels of CrEL prevailing in patients receiving 3 h and 6 h infusion of Taxol® are sufficient for the reversal of the *P*-glycoprotein-mediated MDR in vitro. These plasma levels are not reached during 24 h infusion of Taxol® [16]. Only the unknown MDR-suppressing components of CrEL are captured by this assay making statements concerning the pharmakokinetics of the whole excipient impossible.

The group of Sparreboom developed two methods [18,19] to determine CrEL in plasma of patients under treatment with Taxol®. First, a RP-HPLC-method was established [18], which requires an extended sample preparation including saponification of CrEL in alcoholic KOH, followed by extraction of the released ricinoleic acid with $CHCl_3$ and subsequent derivatisation with 1-naphthylamine. The separation of the resulting products was carried out using Spherisorb ODS-1 as stationary phase and a methanol-acetonitrile-potassium phosphate buffer as mobile phase with UV-detection at 280 nm. Despite of the applicability of this assay proved by pharmakokinetic studies of CrEL in mice and patients receiving Taxol®, only ricinoleic acid containing components of the emulsifier can be monitored, since all other fatty acids cannot be differentiated from the endogenous fatty acids and especially hydrophilic ethoxylated components like glycerol polyoxyethylene ethers or free polyethylene glycol (polyoxyethylene) cannot be captured.

The other method developed by Sparreboom et al. is a colorimetric dye-binding microassay [19] based on rapid binding of Coomassie brilliant blue G 250 to unknown components of CrEL following plasma protein precipitation with acetonitrile and analyte extraction with n-butylchloride. The binding causes a shift in the absorption maximum of the dye from 465 to 624 nm, which is monitored using an automated microplate-absorbance reader.

By this way the pharmakokinetics of CrEL was studied in patients under Taxol® treatment [20]. The results indicate that CrEL is a relatively slow clearance substance which is mainly distributed to the central plasma compartment and not to the tissue, so tumor delivery of the emulsifier is probably insignificant. In consequence, CrEL is not likely to play a role in reversing *P*-glycoprotein-mediated MDR to paclitaxel in solid tumors. This hypothesis correlates with the results of Watanabe et al. [21] who investigated in vivo reversal effects of CrEL in various tumor-bearing mouse models.

In analogy to the HPLC-method [18] the dye-binding microassay with Coomassie blue [19] favoritely captures the hydrophobic portion of the excipient, probably because the more hydrophilic constituents are not coextracted with *n*-butylchloride. Independent of this argument it is unknown which type of the CrEL-components is binding to the dye.

In summary, the major drawback of the methods mentioned above is that none of these techniques is suitable for the simultaneous quantitation of all CrEL-components. Furthermore, the determination of CrEL in plasma of patients receiving Sandimmun® as immunosuppressive drug after kidney-transplantation requires more sensitive methods than the determination in plasma of cancer patients treated with Taxol®, since the applied CrEL-amount is about five times lower.

The potentiometric titration method developed in our group captures all ethoxylated components of CrEL corresponding to 97% of the emulsifier [22]. However, a direct titration of plasma using an anti interference solution AIS is only feasible for plasma levels above 2 mg CrEL per ml because of disturbances by plasma proteins [23].

Table 1
Sorbent materials based on silica gel used for SPE

Unmodified silica Si	(2,3-Dihydroxypropyl) -oxypropyl Diol	Aminopropyl NH_2
Cyanopropyl CN	Cyanopropyl endcappedCN EC	Ethyl C_2
Ethyl endcapped C_2 EC	Octyl C_8	Octyl endcapped C_8 EC
Octadecyl C_{18}	Octadecyl monofunctional C_{18} MF	Octadecyl endcapped C_{18} EC

Since preliminary experiments showed plasma levels in Sandimmun® treated patients to be inferior to this limit of detection, sample preparation is necessary for the separation of CrEL from the disturbing components.

In this paper, both the solid phase extraction (SPE) and the plasma protein precipitation (PPP) will be discussed as suitable procedures.

2. Materials and methods

2.1. Chemicals and materials

CrEL was a gift from BASF AG (Ludwigshafen, Germany). Barium chloride of suprapurgrade was obtained from Merck KGaA (Darmstadt, Germany). Sodium tetraphenylborate (Na-TPB) of analytical-grade was purchased from Acros Inc. (New Jersey, USA). HPLC grade methanol (MeOH) and acetonitrile (MeCN) were purchased from Fluka Chemie AG (Buchs, Switzerland).

Whole blood was collected with 10 ml EDTA-Monovetten® (Sarstedt, Berlin, Germany).

A home-made evaporator was used for the elimination of solvent from samples.

SPE was carried out with IST Isolute columns and the vacuum workstation IST VacMaster, both from Separtis GmbH (Grenzach-Whylen, Germany). If not otherwise indicated, columns with 100 mg sorbent material and 1 ml reservoir volume were used.

The titration apparatus Titrino SM 702 and the non-ionic NIO-Tensid-electrode 6.0507.010 were employed for the quantitation of CrEL using the TiNET 2.4 software for the evaluation of the titration curves (all components from Metrohm AG, Herisau, Switzerland).

2.2. CrEL spiked plasma

Plasma was obtained from whole blood of one of the authors, which was centrifuged for 30 min at 5000 rpm and then stored at ca. -20°C until use. After evaporating 7.0 ml of a methanolic CrEL-solution ($c = 1.0$ mg mL^{-1}) at 70°C under nitrogen stream the CrEL-residue was solved in 7.0 ml plasma.

2.3. Plasma of patients

Whole blood of patients was gained directly after the 4 h-infusion of Sandimmun® and centrifuged in analogy to the whole blood of the volunteer.

2.4. Solid phase extraction SPE

A total of 12 sorbent materials of different polarity ranging from hydrophilic unsubstituted silica to completely hydrophobic RP18-endcapped (EC) modified silica (see Table 1) has been examined referring to the retention behaviour of CrEL. While hydrophilic silica was always conditioned with 1 ml H_2O only (*cw*) and the apolar materials (C_8, C_{18}) with 1 ml MeOH followed by 2 mL H_2O (*cmw*), the intermediate types (Diol, NH_2, C_2, CN) were pre-treated either with MeOH and consecutively with H_2O or with H_2O only in order to optimise column conditioning.

In normal phase adsorption mode unmodified silica is usually conditioned with an apolar solvent like hexane and not with H_2O in order to avoid deactivation of the sorbent. In this work however, a more complex retention mechanism is postulated demanding for this untypical conditioning step (see chapter 3.2.). Furthermore, the inmiscibility of an apolar solvent with aqueous biofluids prevents the conditioning with hexane.

Table 2
Titration parameters with optimised values

Type of regulation	Monotone equivalent point titration
Indication mode	Potentiometric
Temperature	Ambient
Start delay	30 s
Volume increment	0.050 ml
Flow rate of the titrant	1.0 ml min^{-1}
Drift	5 mV min^{-1}
Waiting period	360 s
EP criterion	15 mV
Titrant	1×10^{-3} M Na-TPB-solution
Activator	0.1 M $BaCl_2$-solution

The sorbent materials were tested first with 1.0 ml of an aqueous solution containing 1.0 mg CrEL, then with 1.0 ml of CrEL-spiked plasma of the same concentration. After application of the sample, the column was rinsed with 1 ml H_2O. The retained CrEL-components were eluted with 4 ml MeOH, the methanolic eluate was evaporated, the residue dissolved in water and titrated.

In order to ensure a recovery rate of 100% the purge water was also titrated, when an aqueous solution of CrEL was used. This was impossible with plasma samples, since plasma components cause disturbances.

2.5. Plasma protein precipitation PPP

Protein precipitation was carried out by adding 6 ml MeCN to 3.0 ml CrEL-spiked plasma. After vortexing for 30 s and consecutive centrifugation at 4000 rpm for 15 min the supernatant was separated. The precipitated proteins were washed three times with 2 ml MeCN each. After combining all four supernatants the solvent was evaporated and the residue dissolved in 3.0 ml H_2O.

An aliquot of 1.0 ml of the preceding solution was either titrated directly or after submission to SPE. The final calibration of the determination of CrEL in plasma is based on 2.0 instead of 1.0 ml of the aqueous solution obtained after PPP in order to achieve an improved limit of detection and quantitation.

2.6. Titration procedure

The development of the titration method including preparation of the activator- and titrant-solution has been described in our previous work [22]. Titration parameters are shown in Table 2.

3. Results and discussion

3.1. Titration method

The titration method is based on the activation of the non-ionic emulsifier: Ethoxylated CrEL-components are able to form crown-ether-like complexes with Ba^{2+}- and other mono- and divalent cations [24], so called *pseudocations* stabilised by ion–dipole interactions.

These activated pseudocations can be precipitated with the Na-TPB containing titrant as water-insoluble ternary complexes. The end point of the titration is indicated by a TPB-ionsensitive coated-wire electrode which responds to a surplus of TPB-ions. *All components* of CrEL are monitored with this titration method except of the non-ethoxylated genuine castor oil which is contained in less than 3% of the complexe mixture. Furthermore, a differentiation between components of CrEL is possible: The titration curves (see Fig. 2a) are characterised by two points of inflection (EP1; EP2), the first of which represents the hydrophobic and the second one the more hydrophilic components of the excipient. This assignment could be made by titration of two CrEL-fractions gained by preparative cation-exchange chromatography of CrEL with Amberlite® IRC 50 as sorbent material and identification of the fractions by CD-MECC [25] and DE-MALDI-TOF-MS [26].

Compared to previous work of our group the parameters of the titration were optimised to determine minor amounts of the emulsifier: The volume increment was reduced from 0.200 to 0.050 ml and the concentration of the titrant from 2×10^{-3} to 1×10^{-3} M resulting in an improved limit of detection down to 30 μg (= 28.6 nl) of CrEL from formerly 200 μg concerning the determination in an aqueous solution. The calibration

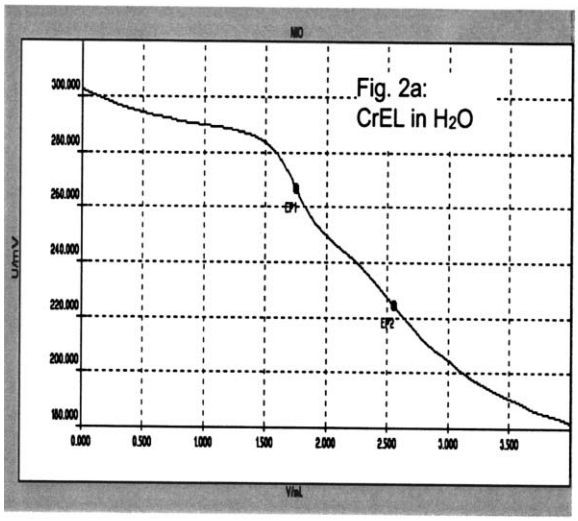


Fig. 2a:
CrEL in H_2O
EP1
EP2
UmV
V/mL

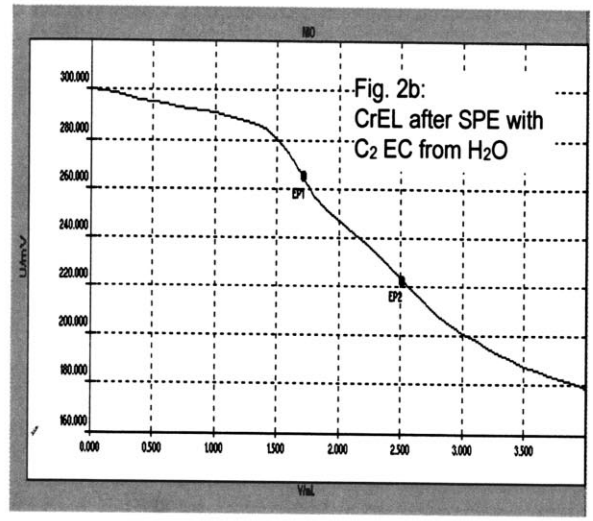


Fig. 2b:
CrEL after SPE with
C_2 EC from H_2O
EP1
EP2
UmV
V/mL

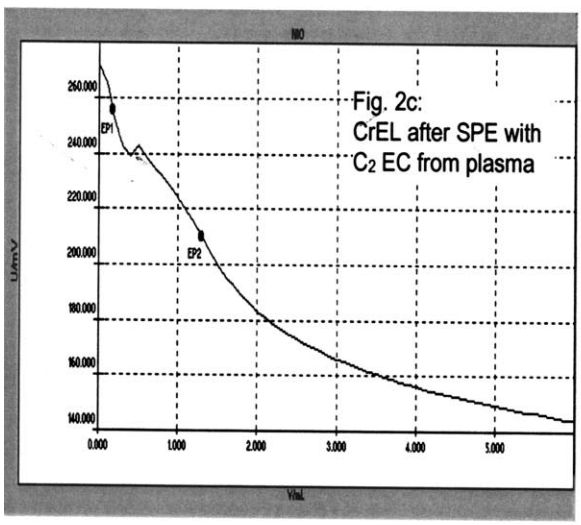


Fig. 2c:
CrEL after SPE with
C_2 EC from plasma
EP1
EP2
UmV
V/mL

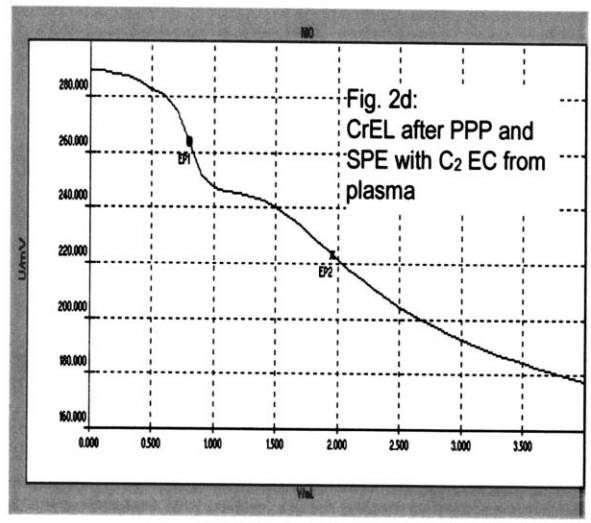


Fig. 2d:
CrEL after PPP and
SPE with C_2 EC from
plasma
EP1
EP2
UmV
V/mL

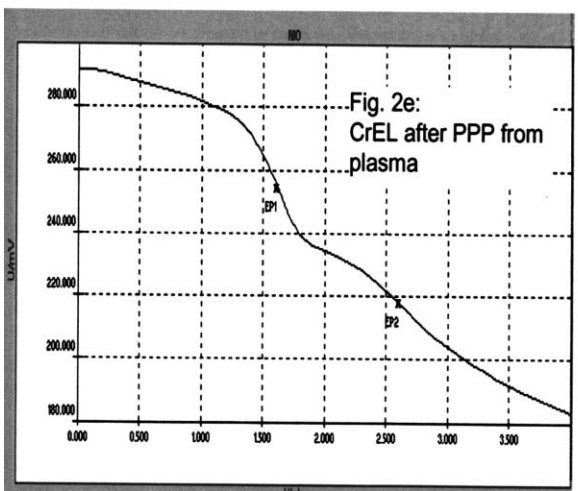


Fig. 2e:
CrEL after PPP from
plasma
EP1
EP2
UmV
V/mL

can be carried out based on EP1 and on EP2, respectively. EP1 was used for this work, because EP2 is only observed with CrEL-amounts down to 300 μg (formerly 750 μg).

3.2. Solid phase extraction SPE

The application of SPE to non-ionic emulsifiers from plasma is not described or proved yet, probably due to their surface activity and their heterogeneous composition. Our work is based on investigations about the adsorption behaviour of ethoxylated alkyl phenols and the corresponding free polyethylene glycol on unmodified silica [27–29].

Using H_2O as solvent the polyoxyethylene ethers show a visibly stronger adsorption compared to that of the free polyethylene glycol because of the minor water solubility of their hydrophobic molecule part and their ability to occupy the silica layer. On the other hand, in MeOH polyoxyethylene ethers do not adsorb at all, since their solubility increases to a significant degree.

In consequence, extraction of CrEL from aqueous solutions using silica as sorbent material and consecutive elution with MeOH should be possible.

3.2.1. SPE from aqueous solutions

Based on the theory mentioned above, first, unmodified Si has been examined with respect to the retention behaviour of CrEL. However, only an incomplete extraction rate of 59.4% was achieved which could merely be improved up to 60.7% using a column with 500 mg of sorbent instead of 100 mg previously used. This result suggests that independent from the column size unmodified Si is not able to adsorb CrEL completely. In consequence sorbent materials with other functional groups and especially of lower polarity have been investigated. In this connection, also the influence of the column conditioning has been studied. The results are summarised in Table 3 and Fig. 3.

The recovery rates of the extraction procedures with any sorbent material obtained as sum of the CrEL amounts from the purge water and from the methanolic eluate were found to be around 100%. With regard to the qualitative composition of the CrEL-components in both the purge water and the eluate most sorbent materials show a similar behaviour: The methanolic eluate contains both the hydrophilic and the hydrophobic components of the emulgent, however, in the purge water only the hydrophobic constituents are found except for the sorbent types CN *cw*, CN *cmw*, C_2(EC) *cw* and C_{18}(EC) *cmw* the purge water of which also contains the hydrophilic components.

The two types of *column conditioning* of the intermediate polar sorbent materials show a different influence on the composition of the corresponding methanolic eluates. These are influenced only in a quantitative way when using CN, CN(EC) and C_2, however, with C_2(EC) as sorbent material, the conditioning step even effects the qualitative composition: Only hydrophobic components are present in the eluate, if the conditioning step is carried out without MeOH.

The *polarity of the sorbent* significantly influences its ability to retain CrEL. An optimal extraction is achieved with materials allowing both polar and apolar interactions with components of the emulsifier: The types CN(EC) *cw*, C_8(EC) *cmw*, C_8 *cmw* and C_2(EC) *cmw* are able to extract more than 90% of CrEL. C_2(EC) *cmw* even yields a nearly complete extraction of 98.2%. Unmodified Si behaves apart from all other materials. Although Si definitely allows only polar interactions, it achieves a better extraction rate than the Diol-, NH_2- and CN-phases which also enable apolar interactions.

Furthermore, endcapping is of prime importance for the adsorption of CrEL-components on the sorbent: The more polar types (CN, C_2) show better results if endcapped, C_{18}, however, achieves

Fig. 2. Titration curves were obtained with 1.0 ml of a CrEL solution. The concentration of the samples (aqueous solution or plasma) before treatment with SPE or PPP, respectively, was 1.0 mg CrEL per ml. Fig. 2a shows the direct titration of an aqueous solution containing 1.0 mg CrEL as reference.

Table 3
Extraction rates of CrEL obtained by SPE from aqueous solution[d]

Sorbent material	EP1 (%) (m/m) [a]	EP2 − EP1 (%) (m/m)	EP2 (%) (m/m) [a]
Reference run	69.4 ± 0.3	30.6	100.0 ± 0.52
Si *cw*[b]	15.8 ± 1.3	43.7	59.4 ± 1.2
Diol *cw*	23.4 ± 0.4	30.6	54.0 ± 0.1
Diol *cmw*[c]	25.8 ± 2.4	28.7	54.6 ± 0.5
NH_2 *cw*	EPs not observed		
NH_2 *cmw*	EPs not observed		
CN *cw*	44.5 ± 1.6	10.2	54.8 ± 0.8
CN *cmw*	42.0 ± 1.0	16.1	58.1 ± 2.2
CN EC *cw*	55.8 ± 0.6	34.6	90.4 ± 0.9
CN EC *cmw*	51.8 ± 4.4	32.7	84.5 ± 0.4
C_2 *cw*	38.0 ± 0.9	37.0	74.9 ± 2.1
C_2 *cmw*	39.5 ± 0.8	40.6	80.0 ± 5.5
C_2 EC *cw*	58.7 ± 4.4	–	EP2 not observed
C_2 EC *cmw*	66.5 ± 2.2	31.8	98.2 ± 1.4
C_8 *cmw*	59.3 ± 5.0	32.8	92.1 ± 2.9
C_8 EC *cmw*	58.7 ± 0.3	33.2	91.9 ± 1.1
C_{18} *cm*w	49.9 ± 0.9	34.0	83.9 ± 1.1
C_{18} MF *cmw*	21.8 ± 0.8	37.7	59.4 ± 0.7
C_{18} EC *cmw*	30.0 ± 1.0	15.8	45.9 ± 2.8

[a] Standard deviations calculated from four determinations.
[b] *cw*, conditioning of the column with 1 ml H_2O.
[c] *cmw*, conditioning of the column with 1 ml MeOH followed by 1 ml H_2O.
[d] Volume of the sample $V = 1.0$ ml; concentration of the sample $c = 1.0$ mg ml^{-1}; mass of the sorbent $m = 100$ mg (unless stated otherwise); elution with 4 ml MeOH (unless stated otherwise).

a significantly reduced extraction rate if endcapped. These results suggest that the more polar types require an endcapping for enhanced apolar interactions, whereas the apolar C_{18} has to be non-endcapped. Probably, the important polar interactions between the sorbent and the components of the excipient are completely suppressed by endcapping a very apolar material.

Some materials, especially Si *cw*, C_2 *cw*, C_2 *cmw* and C_{18} MF *cmw*, show a significant increase of the difference EP2 − EP1. With these materials only an incomplete extraction of the hydrophobic components of the emulsifier is achieved. Probably, other components normally captured at EP1 are now shifted to EP2 due to the loss of interaction with the then missing hydrophobic components. This results in an increase of the difference EP2 − EP1 mentioned above. A similar phenomenon is described in chapter 3.2.2.

Summarising the results, C_2(EC) *cmw* seemed to be the most suitable sorbent material for the extraction of CrEL from aqueous solutions: It achieves the best extraction rate, the titration curve shows no disturbances (see Fig. 2b), a shift of the difference EP2 − EP1 is not observed and the calibration curve is linear with a limit of

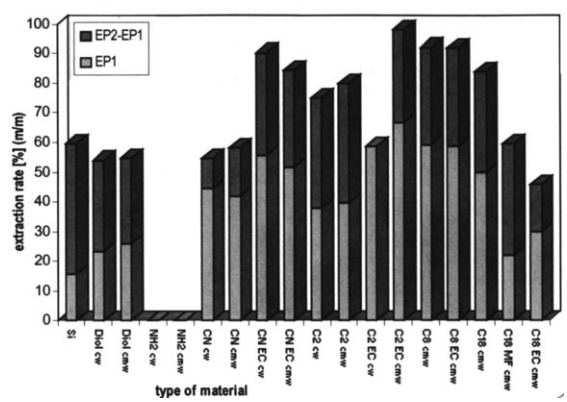


Fig. 3. Extraction of CrEL from aqueous solution by SPE in dependence on the type of sorbent material. Extraction rates were determined with 1.0 ml of an aqueous solution containing 1.0 mg CrEL. For extraction parameters see Table 3.

Table 4
Extraction rates of CrEL obtained by SPE from plasma[d]

Sorbent material	EP1 (%) (m/m)[a]	EP2 − EP1 (%) (m/m)	EP2 (%) (m/m)[a]
Reference run	69.4 ± 0.3	30.6	100.0 ± 0.5
Si *cw* [b]	EPs not observed		
Diol *cw*	4.5 ± 0.9	22.1	26.6 ± 1.5
NH_2 *cw*	EPs not observed		
CN *cmw* [c]	EPs not observed		
CN EC *cw*	4.5 ± 0.7	30.1	34.6 ± 0.8
CN EC *cmw*	4.6 ± 0.4	30.0	34.6 ± 2.1
C_2 *cmw*	5.1 ± 0.6	43.9	49.0 ± 1.7
C_2 EC *cmw*	5.5 ± 1.2	44.7	50.2 ± 1.1
C_2 EC *cmw* 8 mL MeOH	EP1 not observed	–	54.8 ± 1.0
C_2 EC *cmw* 200 mg sorbent	4.4 ± 0.5	52.0	56.5 ± 0.1
C_2 EC *cmw* 500 mg sorbent	12.2 ± 0.7	48.2	60.4 ± 1.0
C_8 *cmw*	EP1 not observed	–	52.0 ± 1.3
C_8 EC *cmw*	5.5 ± 0.4	44.4	49.9 ± 1.3
C_{18} *cmw*	5.2 ± 0.7	38.0	43.2 ± 3.6
C_{18} MF *cmw*	5.9 ± 1.1	43.0	48.9 ± 1.7
C_{18} EC *cmw*	5.4 ± 0.7	–	EP2 not observed

[a] Standard deviations calculated from four determinations.
[b] *cw*, conditioning of the column with 1 ml H_2O.
[c] *cmw*, conditioning of the column with 1 ml MeOH followed by 1 ml H_2O.
[d] Volume of the sample $V = 1.0$ ml; concentration of the sample $c = 1.0$ mg ml^{-1}; mass of the sorbent $m = 100$ mg (unless stated otherwise); elution with 4 ml MeOH (unless stated otherwise).

detection at 70 μg. Thus, C_2(EC) was chosen to explore the extraction of CrEL from plasma.

3.2.2. SPE from plasma

The application of the extraction procedure to plasma using C_2(EC) as sorbent material supplies a significantly lower extraction rate of only 50.2% and a shift of the difference EP2 − EP1 (see Table 4). Besides, the run of the titration is interfered between the two points of inflection (see Fig. 2c) complicating the evaluation especially of EP2. Further investigations showed neither an incomplete elution of the analyte from the column nor an exceeding of the column-capacity which in consequence means that C_2(EC) is not able to extract CrEL completely from plasma. Thus, the other sorbent materials were screened again with respect to their ability to free the emulsifier from plasma. The results (see Table 4 and Fig. 4) show again that none of them achieves a better extraction rate compared to C_2(EC) and none of them improves the shift of the difference EP2 − EP1, respectively. In analogy to the SPE from an aqueous solution, materials which enable polar and apolar interactions simultaneously yield the best results.

The inability of each sorbent material tested to extract more than 50% of the emulsifier and the

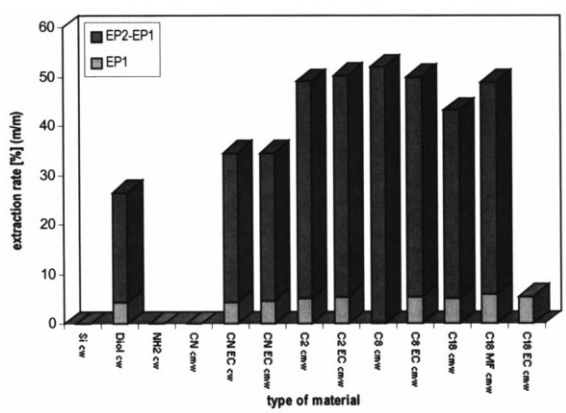


Fig. 4. Extraction of CrEL from plasma by SPE in dependence on the type of sorbent material. Extraction rates were determined with 1.0 ml plasma spiked with 1.0 mg CrEL. For extraction parameters see Table 3.

Table 5
Recovery rates of CrEL obtained by PPP from plasma[b]

Procedure	EP1 (%) (m/m) [a]	EP2 − EP1 (%) (m/m)	EP2 (%) (m/m) [a]
Reference run	69.4 ± 0.3	30.6	100.0 ± 0.5
SPE after PPP (C_2 EC) 100 mg	26.3 ± 3.5	51.5	77.7 ± 2.5
SPE after PPP (C_2 EC) 200 mg	30.1 ± 3.7	62.7	92.9 ± 0.8
SPE after PPP (C_2 EC) 500 mg	44.7 ± 1.0	54.8	99.5 ± 0.2
PPP without consecutive SPE	64.3 ± 1.1	41.5	105.8 ± 1.0

[a] Standard deviations calculated from four determinations.
[b] PPP by adding 6 ml MeCN to 3.0 ml CrEL spiked plasma; after vortexing for 30 s and consecutive centrifugation at 4000 rpm for 15 min separation of the supernatant; washing the precipitated protein fractions three times with 2 ml MeCN each and combining all four supernatants; after evaporation of the solvent and dissolving the residue in 3.0 ml H_2O titration of 1.0 ml of the obtained solution.

significant decrease of EP1 suggest an interaction of especially the hydrophobic components of CrEL with the matrix. Probably, there is a strong binding of the concerned components to plasma proteins. The removal of this hydrophobic portion by protein binding results also in the increase of the difference EP2 − EP1, since other components normally captured at EP1 are now shifted to the EP2.

In consequence of these results, another or additional sample preparation is required for a complete release of CrEL from plasma.

3.3. Plasma protein precipitation PPP

The plasma protein precipitation (PPP) was investigated as a suitable procedure to release the hydrophobic CrEL-components from their plasma protein binding with making them accessible for the SPE. Comparing the different possibilities of protein removal, the precipitation technique with an organic solvent seemed to be advantageous, since the solvent can be easily eliminated again by evaporation. Within the group of solvents used for protein removal MeCN was chosen due to its highest efficiency [30,31].

First, a combination of PPP with MeCN followed by SPE with C_2(EC) as sorbent was examined. The extraction rate significantly increases from 50.2 to 77.7% (see Table 5) and almost no disturbances of the titration curve are observed (see Fig. 2d), however, the shift of the difference EP2 − EP1 is still present. Also, the capacity of the column was investigated with columns of 200 and 500 mg sorbent, respectively, instead of 100 mg normally used, since endogenous substances like bilirubin are released from their protein binding as well which in consequence could lead to an exceeding of the capacity. Indeed, using a column packed with 500 mg sorbent a further increase of the extraction rate to 99.5% is observed (see Table 5). Despite of the almost complete extraction by 500 mg sorbent the shift of the difference EP2 − EP1 is not yet reduced and the disturbances of the titration curve are amplified again.

As an alternative, PPP without following SPE was investigated: An excellent recovery rate of 105.8% is achieved concerning the whole emulgent (EP2) or of 92.6% referring to its hydrophobic components (EP1), (see Table 5). Furthermore, disturbances of the titration curve do not occur with this method (see Fig. 2e) and the shift of the difference EP2 − EP1 decreases.

The method could then be calibrated for the determination of CrEL in plasma based on the PPP as sample preparation. Since the titration of patient samples led to values near to the limit of detection, 2.0 ml of CrEL-spiked plasma instead of 1.0 ml were used. This enlargement of the sample size is possible without increasing disturbances of the titration curve.

The calibration for the determination of CrEL in plasma shows a linear behaviour over the investigated range of concentrations in analogy to the direct titration of an aqueous solution.

The calibration functions are:

$V(titrant)$ [ml] = 1.7569 × m(CrEL) [mg] + 0.0621 for the determination of CrEL in an aqueous solution (correlation coefficient r^2 = 0.9992; standard deviation of the slope sd(b) = 0.0186; standard deviation of the intercept sd(a) = 0.0076).

$V(titrant)$ [ml] = 1.6467 × m(CrEL) [mg] + 0.0651 for the determination of CrEL in plasma after PPP (correlation coefficient r^2 = 0.9962; sd(b) = 0.0386; sd(a) = 0.0157).

The relative standard deviations of the measured points range from 0.4 to 13.5% with 1000 and 40 μg CrEL, respectively (standard deviations were calculated from four determinations). Thus, a sufficient precision for the quantitative determination from a biological matrix is ensured, since the acceptable deviation of 20% is not exceeded in any case.

In analogy to the determination from aqueous solutions, the limit of detection and quantitation was found as 30 μg CrEL in the plasma volume of 2 ml corresponding to a concentration of 15 μg CrEL per ml.

3.4. Determination of CrEL in plasma of patients

The applicability of the developed procedure was investigated by using samples of patients undergoing a Sandimmun® treatment. CrEL was thus quantified in the plasma of patients of different physical constitution and age.

The results (see Table 6) clearly suggest the applicability of the titration method in combination with the preceding PPP. Furthermore, individual variations of the plasma levels are observed: Since patients No.1 and No.3 show similar body masses, the CrEL doses they received were almost identical allowing a direct comparison of their plasma levels of the emulsifier. This comparison shows a significant difference, especially after the first infusion of Sandimmun® indicating an inter individual variation of the distribution of CrEL to the tissues.

These tendencies have to be further investigated within the framework of a complete elimination kinetics.

4. Conclusions

In this work, we have described the development of a procedure for the quantitative determination of CrEL in plasma of patients under Sandimmun® treatment based on a potentiometric titration method with a special type of an ionsensitive electrode.

Sample preparation for the isolation of the excipient is required due to disturbances by plasma proteins and to the low plasma levels of the non-ionic emulsifier. Therefore, the SPE and the PPP were investigated. The sorbent material C_2(EC) used for SPE as sample preparation is able to remove CrEL from an aqueous solution, whereas the extraction from plasma is significantly interfered by a strong plasma protein binding. However, this binding can be broken by PPP with MeCN.

Thus, PPP followed by the potentiometric titration enables the quantitation of CrEL from plasma of patients after kidney-transplantation

Table 6
CrEL-levels in plasma of patients under Sandimmun®-treatment

Age of patient (a)	Body weight of patient (kg)	Body height of patient (cm)	Dose of CrEL per infusion [a] (g)	Plasma level after 1st inf.[b] (g ml^{-1})	plasma level after 2nd inf. [b] (g ml^{-1})
51	71.0	172	2.769	67.1 ± 6.9	55.0 ± 0.7
52	91.0	177	3.549	63.1 ± 1.4	92.1 ± 0.9
40	76.5	180	2.984	28.0 ± 1.3	49.3 ± 1.2
38	80.0	180	3.120	100.7 ± 5.1	EP1 not found

[a] Sandimmun® contains 13.0 mg CrEL per mg ciclosporin. An amount of 3.0 mg ciclosporin per kg body weight is administered during each infusion, which is equal to an application of 39.0 mg CrEL per kg body weight.

[b] Standard deviations calculated from two determinations.

receiving Sandimmun® as immunosuppressive drug. Based on this procedure the pharmakokinetics of the excipient used in this commercial preparation will be investigated.

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