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Plasma assay for the antineoplastic agent VP 16-213 (etoposide) using high-performance liquid chromatography with electrochemical detection

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Abstract: A sensitive high-performance liquid chromatographic (HPLC) assay of the antineoplastic agent VP 16-213 (etoposide) in plasma is described. The system discriminates between the parent compound and possible metabolites, including the aglycone and the *cis* isomer. After extraction with 1,2-dichloroethane the drugs are chromatographed on a reversed-phase phenyl column with amperometric detection. Quantitative response is linear up to 250 ng/ml for 1 ml human plasma and up to 40.0 μg/ml for 0.1 ml human plasma. The detection limit is *ca* 2 ng/ml in plasma. Preliminary pharmacokinetic results show that the sensitivity and selectivity of the assay are adequate to establish plasma concentrations over 8–12 half-lives during elimination of the drug.

Keywords: *High-performance liquid chromatography; anti-tumour agents; etoposide; electrochemical chromatographic detection.*

Introduction

The epipodophyllotoxine derivative VP 16-213 (etoposide) (Scheme **1**) is an antineoplastic agent active against various neoplastic diseases, e.g. small cell anaplastic bronchial carcinoma, testicular teratomas [1, 2]. VP 16-213 is synthesized from podophyllotoxine, a lignane derivative obtained by extraction of the roots of *Podophyllum peltatum*. Podophyllotoxine is epimerized, demethylated at the 4′ position and coupled to a glucopyranoside group at the 4 position by an ether linkage. VP 16-213 is more potent and less toxic than the parent compound [3]. It is highly lipophilic with a molecular weight of 588. Experiments with labelled compounds have shown that 15% of an intravenously administered dose is excreted as metabolites, possibly ring-opened lactones [4, 5] and aglycones (Scheme **2**). Although the compound has been extensively studied in clinical trials, its metabolism is poorly understood and is receiving increasing attention [6].

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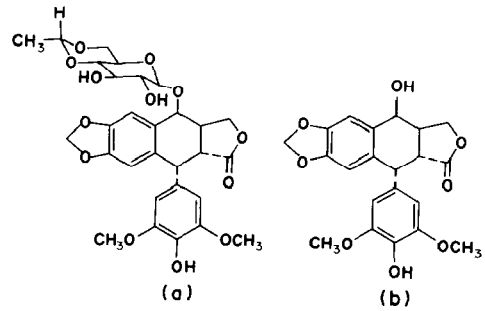
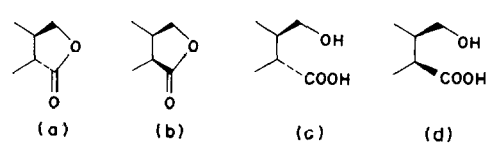
Scheme 1
Structure of (a) VP 16-213 and (b) the aglycone of VP 16-213.



The extraction of VP 16-213 from biological fluids is complicated by the instability of the compound and its metabolites at extreme pH values [4]. For the determination of VP 16-213 in plasma, an assay is needed which can discriminate between the *trans*-VP 16-213 administered and *cis*-VP 16-213 (a possible inactive metabolite and decomposition product), and between the *cis* and *trans* isomers of the hydroxy acid of VP 16-213. There is some evidence that the *trans* isomer of the hydroxy acid of VP 16-213 is the major metabolite [4, 5]. However, this compound has not yet been synthesized due to epimerization of the lactone ring to the *cis* configuration at pH values above 9.5 [4]. Current methods for the analysis of VP 16-213 employ solvent extraction followed by HPLC with UV [4, 7–9] or fluorimetric [10] detection. However, the low UV absorptivity and fluorimetric intensity of the epipodophyllotoxins lead to poor detection limits for VP 16-213 with these methods (30 ng/ml for UV detection [9] and 50 ng/ml for fluorimetric detection [10]).

Scheme 2
Configurations of the lactone moiety of VP 16-213, metabolites and degradation products. (a) VP 16-213 (*trans*-VP 16-213); (b) *cis* VP 16-213; (c) *trans*-hydroxy acid of VP 16-213 (or aglycone); (d) *cis*-hydroxy acid of VP 16-213 (or aglycone).



Electrochemical detection offers a sensitive detection method in liquid chromatography and has received much attention for the analysis of pharmaceuticals in biological fluids. VP 16-213 and its metabolites and decomposition products contain a phenolic hydroxyl group which is electrochemically active. This paper presents an assay for VP 16-213 using HPLC with electrochemical detection, which permits quantitation at levels lower than those previously reported. The method has been employed to determine plasma levels after intravenous administration of VP 16-213 to cancer patients. It permits the selective analysis of VP 16-213 in the presence of its metabolites and degradation products. These compounds have not been quantitatively determined because of the lack of knowledge of their clinical and pharmacological importance. Preliminary pharmacokinetic data are also presented.

Experimental

Reagents

VP 16-213 and its aglycone were kindly supplied by Bristol Myers Nederland B.V. The

cis-hydroxy acid of the VP 16-213 aglycone and the *cis*-hydroxy acid of VP 16-213 were synthesized using the method of Strife *et al.* [4]. Solvents and chemicals were of analytical grade and used without further purification. All solutions were prepared with deionized water.

Apparatus

The chromatographic system consisted of a 6000 A solvent delivery system and a U 6 K septumless injector (Waters Associates, Milford, MA, USA). An empty HPLC column was placed between pump and injector to reduce pump pulsations. The flow rate was 1.0 ml per min. The analytical column (30 cm × 4.6 mm i.d.) was slurry packed with 10 μm μBondapak Phenyl material. The electrochemical detector cell employed was of the centrally injected type developed at our laboratory and was connected to a Metrohm 641 VA potentiostat. The cell consisted of a glassy carbon working electrode (Metrohm EA 286/1, diameter 5 mm) placed in the centre of a platinum ring auxiliary electrode (inside diameter 10 mm, outer diameter 13 mm). The distance between the glassy carbon electrode and the jet could be adjusted by a micrometer on which the electrode was mounted. An Ag/AgCl reference electrode with a Vycor® glass-tipped jacket was placed close to the working and the auxiliary electrode. The diameter of the outlet jet of the eluent of the chromatographic system was 0.11 mm. The cell was submerged in a vessel containing the mobile phase. (A detailed description of this sensitive and low cost electrochemical HPLC detector is available from the authors on request.) The whole detector was shielded to prevent electrical disturbance. The detector and both columns were thermostatted at 24°C. For the assay of VP 16-213 in plasma, the mobile phase consisted of methanol/0.065 M phosphate buffer (pH 7.0) (60:40 w/w). For the separation of possible metabolites and degradation products, the mobile phase consisted of methanol/0.065 M phosphate buffer (pH 7.0) (40:60 w/w). The mobile phases were filtered by a millipore filter (0.2 μm) and degassed by sonication for 15 min. The glassy carbon electrode was cleaned daily by polishing with 0.3 μm Al_2O_3 powder (Metrohm EA 1086).

Procedures

Sample treatment. For the analysis of VP 16-213 in plasma at concentrations above 250 ng/ml, 1.0 ml of 1,2-dichloroethane (DCE) was added to 100 μl plasma in a polypropylene tube (4 ml). For the analysis of concentrations below 250 ng/ml, 1.0 ml of DCE was added to 1.0 ml of plasma. After shaking for 1 min the extraction mixture was centrifuged. The organic layer (0.8 ml) was removed and evaporated at 30°C under nitrogen. The residue was redissolved by sonication for 6 min in 25–100 μl of the mobile phase and 5–20 μl was injected onto the chromatograph. Standard plasma samples for calibration were prepared by spiking plasma (free of VP 16-213) and were processed with each set of patient samples. Blood samples were collected from patients during the 30–60 min period of intravenous infusion of VP 16-213 (in a 2- or 3-day schedule) and for 1–3 days after the last dose. Immediately after the blood had been taken in heparin-containing tubes, the samples were centrifuged and the plasma transferred to a glass tube and stored at −18°C. For the analysis of patients' plasma samples, each sample was extracted twice and, if possible, two chromatographic runs were performed on each redissolved extract.

Results and Discussion

Hydrodynamic voltammetry

VP 16-213 (10^{-4}M in mobile phase) shows in linear-sweep voltammetry two oxidative waves (E_{p1} = +370 mV, E_{p2} = +690 mV vs Ag/AgCl reference electrode). This voltammetric activity is caused by the oxidation of the phenolic hydroxyl group, which in the middle pH range proved to be a two-electron process. (A more detailed study on the oxidation mechanism of VP 16-213 and its metabolites is in progress). Figure 1 shows the

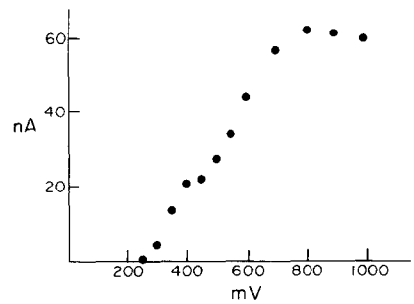


Figure 1
Hydrodynamic voltammogram after injection of 200 ng VP 16-213.

current–potential curve of VP 16-213 obtained by hydrodynamic voltammetry using the chromatographic peak current. It can be seen that a first plateau was reached at +400 mV and a second one at +800 mV, correlating with the first and the second electron transfer respectively. Because of the high lipophilicity of VP 16-213, relatively high concentrations of methanol in the mobile phase were necessary to elute VP 16-213 compared with many aromatic hydroxides and amines, e.g. adrenergic amines and catecholamines. This prevented the use of electrode materials with a low background current such as carbon paste. The higher background current and noise levels of the glassy carbon electrode [11] made it necessary to optimize the potential for the detection of VP 16-213 in this system. The relationship between the potential of the glassy carbon working electrode, the background current and the noise is shown in Fig. 2. The

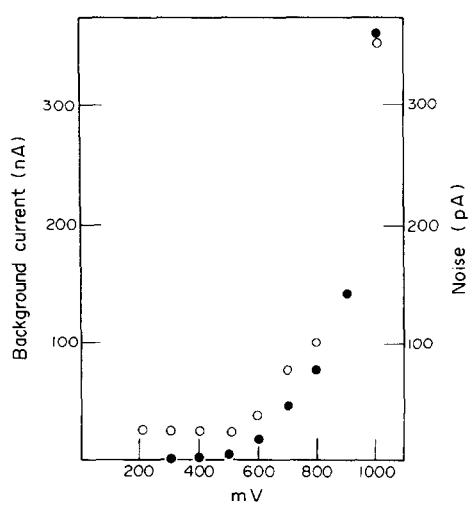


Figure 2
Noise and background current versus potential of the glassy carbon electrode. ● Background current: ○ noise.

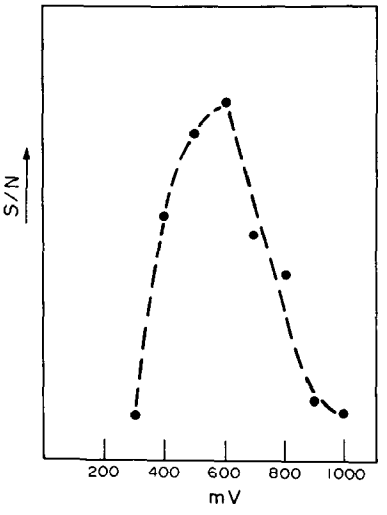


Figure 3
Signal-to-noise ratio versus potential of the glassy carbon electrode (1 ng VP 16-213 injected).

relationship between potential and signal-to-noise ratio showed a maximum at +600 mV (Fig. 3). At higher potentials the background current increased exponentially with peak height. Figure 2 shows that up to a potential of +500 mV the absolute values of the background current and the noise were relatively low. Despite the fact that only one-electron transfer is exploited in the 500–600 mV range, the maximum in Fig. 3 occurs in this region; a potential of +500 mV was thus chosen to detect VP 16-213. The low background current obtained also contributed to the linearity of the detector and to the short stabilization period after polishing the working electrode. A minimal stabilization period after polishing was achieved by polarizing the electrode in the detector cell at a higher potential. The flow rate was set at 1.0 ml/min and the potential adjusted to +700 mV for 10 min; the potential was then decreased to +500 mV. This procedure resulted in a background current lower than 10 nA within 20 min. A further decrease was observed over 1 h until a stable background current of 3 nA was reached.

Calibration, linearity and reproducibility of the detector

Calibration curves of VP 16-213 dissolved in the mobile phase were linear in the range 0.5–1000 ng injected. The correlation coefficient was 0.9997 and the intercept was not significantly different from zero ($p = 0.95$). The wide linear range of the detector was attributable to the low resistance between the electrodes and the low background current, resulting in a low IR drop even in the rather apolar mobile phase. The IR drop is the loss of voltage of the working electrode due to the resistance between working electrode, auxiliary electrode and reference electrode. The within-day relative standard deviation (RSD) for repetitive determinations of 100 ng VP 16-213 dissolved in the mobile phase was 1.7% ($n = 10$). The RSD for repeated determinations over 4 days of 100 ng VP 16-213 in the mobile phase was 4.55% ($n = 6$). The detection limit of VP 16-213 in this system was 250 pg (425 fmol) based on a signal-to-noise ratio of 3. This sensitivity is an order of magnitude greater than that reported for HPLC [9, 10].

Chromatography of VP 16-213, degradation products and metabolites of VP 16-213

Using a reversed-phase C-18 analytical column it was not possible to resolve VP 16-213

and the *cis*-isomer at any concentration of methanol in the mobile phase. A phenyl reversed-phase column, however, was able to discriminate between VP 16-213, the *cis*-isomer of VP 16-213, and the *cis*-hydroxy acid of the aglycone of VP 16-213 under optimum chromatographic conditions. Figure 4 shows a typical chromatogram after

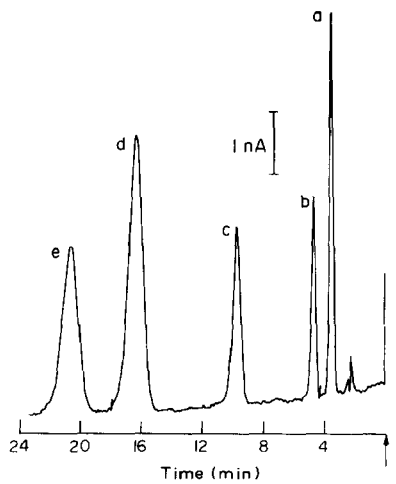


Figure 4
Chromatogram of a mixture of epipodophyllotoxine derivatives. (a) *cis*-hydroxy acid of the aglycone of VP 16-213 (52 ng); (b) *cis*-hydroxy acid of VP 16-213 (50 ng); (c) aglycone of VP 16-213 (100 ng); (d) VP 16-213 (250 ng) *trans* form); (e) *cis*-VP 16-213 (250 ng). Mobile phase: methanol/0.065 M phosphate buffer (pH 7.0) (40:60 w/w).

injection of a mixture of these compounds, using the eluent containing 40% w/w methanol. It was not possible to obtain baseline resolution between VP 16-213 and the *cis*-isomer using the 60% w/w methanol eluent, although the difference in retention time was sufficient to distinguish the two compounds qualitatively.

Analysis of plasma

Chloroform, dichloromethane, ethylacetate and 1,2-;dichloroethane (DCE) were all found to be suitable extraction solvents for VP 16-213 (extraction coefficient > 50). DCE was chosen because of its greater purity, leading to fewer interfering peaks from plasma in the chromatogram. Because of the adsorption of VP 16-213 onto glass, polypropylene tubes were used. As can be seen from Table 1, the extraction yield approaches 100% with an acceptable relative standard deviation in both concentration ranges. Calibration

Table 1
Recoveries and relative standard deviations of analysis of VP 16-213 in spiked plasma samples

Experiment	Sample volume (ml)	Recovery (%)	RSD (%)	*n*
1*	0.1	98.5	3.1	8
2†	1.0	97.8	3.6	5
3‡	0.1	-	2.8	7

* All samples were obtained from one batch of plasma and were spiked with 500 ng VP 16-213.
† All samples were obtained from one batch of plasma and were spiked with 50 ng VP 16-213.
‡ Repeated analysis of 0.1 ml plasma from one patient, after administration of VP-213.

curves of VP 16-213 in plasma in the range 2–250 ng/ml were linear, with a slope of 0.1289, an intercept of −0.031 and a correlation coefficient ($p = 0.95$) of 0.9998. In the range 2.5–40.0 μg/ml plasma (where the sample size was 0.1 ml) the slope was 0.1568, the intercept +0.920 and the correlation coefficient 0.9997. The intercepts were not significantly different from zero ($p = 0.95$).

Figure 5 shows typical chromatograms of blank and patient plasma samples. With the

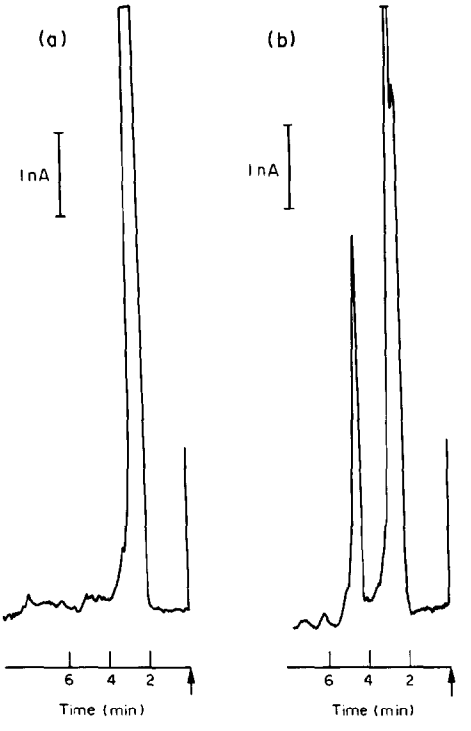


Figure 5
(a) Chromatogram of a blank plasma extract (1.0 ml). (b) Chromatogram of an extract of patient plasma (1.0 ml) containing VP 16-213 at 190 ng/ml. Mobile phase: methanol/0.065 M phosphate buffer (pH = 7.0) (60:40 w/w).

procedure employed, only VP 16-213, *cis*-VP 16-213 and the aglycone of VP 16-213 were extracted. For the analysis of the hydroxy acids the development of ion-pair extraction procedures should be feasible, since at the pH of plasma both the hydroxy acids of VP 16-213 and the hydroxy acids of the aglycone of VP 16-213 are ionized.

In agreement with Scalzo *et al.* [12] and Strife *et al.* [4] and contrary to the work of Evans *et al.* [13], this study did not detect *cis*-VP 16-213 in any plasma sample. This suggests that *cis*-VP 16-213 could be an artefact. The other extracted metabolite, the aglycone of VP 16-213, was not retained on the column with a mobile phase containing 60% w/w methanol.

Routine analysis

The greatest drawback of solid glassy carbon electrodes is the contamination of the electrode surface due to adsorption of oxidation products and the lipophilic parent compound, which causes a loss in activity of the electrode. The adsorption process is more pronounced during the oxidation of compounds with high molecular weights and a more lipophilic character, when the oxidation products are only slowly desorbed from the electrode surface. This adsorption problem interferes substantially when a series of

plasma samples is studied with large differences in individual concentrations. The centrally-injected detector-cell developed in this work tackles these problems by using a unique geometry whose features are a very small jet diameter, an adjustable distance from the jet to the glassy carbon electrode, and compactness. The detector behaves as a wall-jet cell or as a thin layer cell, depending on the distance between the jet and glassy-carbon electrode [14, 15]. This results in short residence times of lipophilic compounds at the electrode surface and in fast and almost complete desorption of these compounds and oxidation products during the analysis. To limit the decrease in activity of the electrode, it was important that the injected amounts of oxidizable compounds should be minimized. To obtain reliable results it was also necessary to determine the calibration status regularly during a long series of samples. This was performed by injecting 100 ng VP 16-213 after each 6 injections of plasma extracts in bracketting sequence. The resulting signals were used to correct the peak heights for the plasma extracts. The sensitivity of the working electrode decreased by up to 20% over one day, if plasma samples were analysed continuously: this bracketting correction procedure thus proved to be more practical than repetition of the polishing procedure. The correction method proposed was preferred to the use of an internal standard, as no compound was available with comparable lipophilicity, chromatographic extraction and electrochemical properties. The structural analogue VM 26 failed in this respect due to the presence of a thiophene ring instead of a methyl group.

Preliminary pharmacokinetic results

To prove its applicability the method described was tested on patients' blood samples after intravenous administration of VP 16-213 in a pharmacokinetic study; typical results for two patients are reported. Patient A received 120mg/m^2 VP 16-213 over 60 min by i.v. infusion on day 1 and day 2, and over 120 min by infusion on day 3. In Fig. 6 the log (plasma concentration) vs time curve after each administration fits a two-compartment model with elimination half-lives ($t_{1/2}$) of 5.2, 7.5 and 8.0 h. Because of the lack of satisfactory data, the $t_{1/2}$ of the second infusion is uncertain. Patient B received

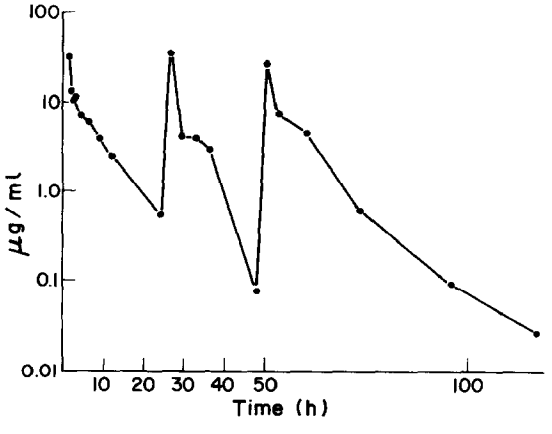


Figure 6
Log (plasma concentration) versus time curve after administration of VP 16-213 to patient A (for details, see text).

120 mg/m^2 over 60 min by i.v. infusion in a two day schedule. The plasma samples obtained from this patient were assayed twice. The plasma concentration levels obtained in the two experiments were statistically evaluated and were found to be not significantly different ($p = 0.95$). The pharmacokinetic curve of patient B after each administration fitted a two-compartment model with elimination half-lives of 15.4 and 14.7 h. A more detailed study of the clinical pharmacokinetics of VP 16-213 is currently being undertaken.

The method described in this paper has proved to be selective for VP 16-213 in all therapeutic regimens used in modern clinical therapy, i.e. single agent combination regimens, as well as co-administration with additional drugs (e.g. analgesics). The present method can readily be used in clinical pharmacological experiments, and provides an opportunity to investigate individual variations in the pharmacokinetics of the drug, due to its selective and sensitive detection of metabolites.

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References

[1] M. Rozencweig, D. D. Von Hoff, J. E. Henny and F. M. Muggia, *Cancer* **40**, 334–342 (1977).
[2] A. M. Arnold, *Cancer Chemother. Pharmacol.* **3**, 71–80 (1979).
[3] C. Keller-Justén, M. Kuhn, A. von Wartburg and H. Stähelin, *J. Med. Chem.* **14**, 936–940 (1971).
[4] R. J. Strife, I. Jardine and M. Colvin, *J. Chromatogr.* **182**, 211–220 (1980).
[5] L. M. Allen, C. Marcks and P. J. Creaven, *Proc. Am. Assoc. Cancer Res.* **17**, 6 (1976).
[6] J. M. S. van Maanen, W. J. van Oort and H. M. Pinedo, *Eur. J. Cancer Clin. Oncol.* **18**, 885–890 (1980).
[7] P. Farina, G. Marzillo and M. d'Incalci, *J. Chromatogr.* **222**, 141–145 (1981).
[8] L. M. Allen, *J. Pharm. Sci.* **69**, 1440–1441 (1980).
[9] J. J. M. Holthuis, H. M. Pinedo and W. J. van Oort, *Anal. Chim. Acta* **130**, 23–30 (1981).
[10] R. J. Strife, I. Jardine and M. Colvin, *J. Chromatogr.* **224**, 168–174 (1981).
[11] K. Stulik and V. Pacáková, *J. Chromatogr.* **208**, 269–278 (1981).
[12] A. J. Scalzo, R. Comis, A. Fitzpatrick, B. F. Issell, P. A. Nardella, M. Pfeffer, R. D. Smyth and D. R. Harken, *Proc. Am. Soc. Clin. Oncol.* **18**, 129 (1982).
[13] W. E. Evans, J. A. Sinkule, A. Horváth, W. R. Crom, L. W. Dow and G. Rivera, *Proc. Am. Assoc. Cancer Res.* **22**, 174 (1981).
[14] J. Yamada and H. Matsuda, *J. Electroanal. Chem.* **44**, 189–198 (1973).
[15] W. P. van Bennekom, Ph.D. Thesis, University of Leiden, The Netherlands (1981).

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