ELSEVIER

Journal of Chromatography A, 711 (1995) 289–295

JOURNAL OF CHROMATOGRAPHY A

Quantitation of digitoxin, digoxin, and their metabolites by high-performance liquid chromatography using pulsed amperometric detection

Kevin L. Kelly*, Bruce A. Kimball, John J. Johnston

U.S. Department of Agriculture, Animal and Health Inspection Service, Denver Wildlife Research Center, Building 16, Denver Federal Center, Denver, CO 80225, USA

First received 24 January 1995; revised manuscript received 25 April 1995; accepted 1 May 1995

Abstract

A novel detection method for the cardiac glycosides digitoxin, digoxin, and their metabolites is described. Both isocratic and gradient water–acetonitrile elutions were employed with an octadecylsilane column. The effluent was monitored by integrated pulsed amperometry in an alkaline medium (pH > 12) by adding sodium hydroxide post-column. Using isocratic elution, peak responses were linear from 10 to 70 ng, with lower limits of detection between 4 and 15 ng. This method allows easy modification of well-established high-performance liquid chromatographic systems, as well as comparable sensitivity and greater selectivity than UV detection.

1. Introduction

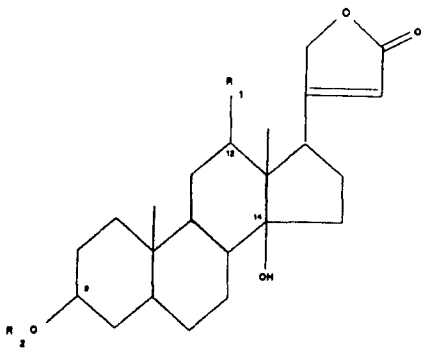
Digitoxin and digoxin are secondary cardiac glycosides which occur naturally in the foxglove plant (i.e. *Digitalis lanata* and *Digitalis purpurea*) through enzymatic conversion of their respective primary cardiac glycosides, lanatoside A and C. These compounds are widely used in the therapeutic treatment of heart disease. It is well known that cardioactive metabolites can be produced from these glycosides by stepwise cleavage of the three digitoxose sugars attached to the aglycone steroid at the C-3 atom [1,2]. The major metabolic products are digitoxose, mono- and bisdigitoxosides, and the remaining aglycone steroid, either digitoxigenin or digoxigenin (Table 1). In some cases, the metabolites are more toxic than the parent compounds [3].

A wide variety of chromatographic methods have been used for the separation of cardiac glycosides, including gas (GC) [4–6], thin-layer (TLC) [7–9], and high-performance liquid chromatography (HPLC) [10–16]. The GC procedures require hydrolysis to yield the corresponding aglycone, which is subsequently derivatized for greater volatility. Although these procedures employ sensitive GC detectors, the determination of individual cardiac glycosides within the same cardenolide series is not possible because they all contain the same aglycone. The TLC methods generally suffer from a lack of sensitivity, long development times, poor resolution, or require long post-separation conditioning times for more sensitive fluorescence detection [7]. High-performance liquid chro-

* Corresponding author.

SSDI 0021-9673(95)00525-0

Table 1
Structures of the cardiac glycosides and aglycones



Compound	R_1	R_2^a
Digitoxin	H	-D-D-D
Digitoxigenin bisdigitoxoside	H	-D-D
Digitoxigenin monodigitoxoside	H	-D
Digitoxigenin	H	H
Digoxin	OH	-D-D-D
Digoxigenin bisdigitoxoside	OH	-D-D
Digoxigenin monodigitoxoside	OH	-D
Digoxigenin	OH	H

[a] D = D-digitoxose (2,6-dideoxy-ribo-hexose).

matographic separations of cardiac glycosides involve less labor, and yield fast, reproducible results. However, the cardiac glycosides contain a relatively weak UV chromophore with λ_{max} at about 220 nm. Many organic compounds absorb at this wavelength and will interfere if they are not adequately separated from the cardiac glycosides. In addition, mobile phase solvents with high transparency at 220 nm must be used in order to achieve low detection limits. Pre- or post-column derivatization is necessary to achieve more selective and sensitive UV or fluorescence detection [17,18].

This work focuses on the use of pulsed amperometric detection (PAD) for the quantitation of the cardiac glycosides and their corresponding aglycones. This method was investigated for the detection of the sugar moieties of the cardiac glycosides because of the success of previous works using PAD for the detection of carbohydrates [19–23]. Furthermore, pulsed amperometric detection allows specific electrochemical detection without derivatization. Carbohydrates are detected by direct oxidation on a gold working electrode under alkaline conditions. Standard high-performance liquid chromatographic systems can be easily modified to permit PAD detection of the cardiac glycosides and their aglycones.

2. Experimental

2.1. Instrumentation

A Hewlett-Packard 1090M liquid chromatograph equipped with a computer workstation (Hewlett-Packard, Palo Alto, CA, USA) was used for the separation of digitoxin, digoxin, and their metabolites. The octadecylsilane analytical column measured 250 × 4.6 mm I.D. (Deltabond, Keystone Scientific, Bellefonte, PA, USA).

The chromatograph was equipped with a pulsed electrochemical detector (Dionex Corp., Sunnyvale, CA, USA) operated in the integrated amperometry mode.

A gradient pump (Dionex, series 4500i) was used to deliver 1.0 *M* sodium hydroxide post-column. The electrochemical cell consisted of an organic solvent compatible gold working electrode (1.4 mm), a stainless steel counter electrode, and a Ag/AgCl reference electrode. The gasket, which permits flow across the working electrode, was 0.005 inch thick. The detector analog signal was inputted to the workstation via a multichannel interface (HP 35900, Hewlett-Packard).

2.2. Reagents

Digitoxin, digitoxigenin, digoxin, and digoxigenin were obtained from Sigma (St. Louis, MO, USA). Digoxigenin monodigitoxoside was provided by Burroughs Wellcome Co. (Research Triangle, NC, USA). All other mono- and bisdigitoxosides were obtained from Indofine Chemical Co. (Belle Mead, NJ, USA). All analytes were of analytical grade and used with-

out further purification. The molecular structures of these compounds are given in Table 1. Stock solutions of each cardiac glycoside and aglycone were prepared separately in HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ, USA). The stock solutions were kept in the dark and refrigerated at 4°C. No degradation of the analytes was observed over 30 days when the stock solutions were in use. These stock solutions were then used to prepare separate and mixed diluted working standards of the cardiac glycosides on the day of the chromatographic experiments.

HPLC grade acetonitrile (ACN, Fisher Scientific) was filtered once through a 0.45-μm nylon filter before use. Water was purified using the Milli-Q + purification system (0.22 μm, Millipore, Molsheim, France). Concentrated 50% w/w sodium hydroxide (NaOH) was obtained from J.T. Baker (Phillipsburg, NJ, USA) and diluted with water to a concentration of 1.0 *M*. All solvents were degassed with industrial grade helium (Airco Gases, Denver, CO, USA). A helium headspace was maintained in the NaOH reservoir to prevent absorption of carbon dioxide.

2.3. Procedure

Separation of each cardenolide series was done under isocratic conditions. The mobile phase composition was water–ACN (67:33, v/v) for the digitoxin cardenolide series, and water–ACN (77:23, v/v) for the digoxin cardenolide series. For the separation of a combined mixture of both the digitoxin and digoxin cardenolide series, a linear gradient elution from water–ACN (90:10) to water–ACN (55:45) over 8 min was performed. The HPLC flow-rate was 1.0 ml/min for isocratic elution and 1.3 ml/min for gradient elution. The post-column addition of 1.0 *M* NaOH was done at an optimum flow-rate of 0.5 ml/min. A 500-μl reaction coil (Dionex) was used after the tee to ensure uniform mixing prior to the detector.

For PAD detection, the applied potentials and their durations were as followed: $E_1 = +0.07$ V, $t_1 = 400$ ms; $E_2 = +0.70$ V, $t_2 = 120$ ms; $E_3 = -1.00$ V, $t_3 = 300$ ms. The duration of the sampling period was 300 ms. The analog full-scale range was 100 nanocoulomb (nC) for isocratic elution and 300 nC for gradient elution.

The gold working electrode was lightly polished approximately every 200 h to remove oxidized materials. No degradation in the reproducibility or the stability of the gold working electrode was observed over a period of 9 months.

Calibration curves were constructed using the mean peak areas from triplicate chromatograms under the same isocratic conditions as discussed previously. A minimum of 4 standard solutions within the range of 10–70 ng/ml were used for each calibration curve.

To measure the effect of the concentration of ACN in the mobile phase on peak area, the column was replaced with a zero dead volume union connector and injections of digoxigenin monodigitoxoside were made. Small aliquots (100 μl) of the stock solution of the compound were evaporated under nitrogen and reconstituted in the same water–ACN solution as used for the HPLC mobile phase. The reconstituted solutions were further diluted to 1.0 ml each to achieve a final concentration of 48.9 μg/ml. Triplicate injections were made in the order of increasing % ACN composition at 10% increment intervals from 10% to 90%. The flow-rates for the HPLC and IC pumps were 0.6 ml/min and 0.3 ml/min, keeping the same flow-rate ratio as in the isocratic elutions. All other parameters remained the same.

3. Results and discussion

3.1. Chromatography

A representative chromatogram is shown in Fig. 1 for the digoxin cardenolides series using an isocratic mobile phase composition. In addition, separation of all 8 cardenolides compounds from both the digitoxin and digoxin series during gradient elution is shown in Fig. 2. The decreasing drift in the baseline in Fig. 2 is due to adsorption of ACN on the surface of the gold working electrode, hindering detection of the

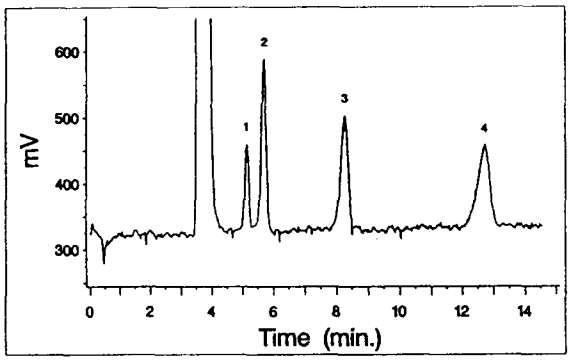


Fig. 1. Isocratic separation of a mixed standard of digoxigenin (1), digoxigenin monodigitoxoside (2), digoxigenin bisdigitoxoside (3), and digoxin (4) obtained by reversed-phase HPLC; 50 pmole of each compound injected in 10 μl of methanol. The mobile phase was water–ACN (77:23) at a flow-rate of 1.0 ml/min. The large unlabeled peak at ± 4 min is methanol.

analytes [22,24]. The chromatograms presented here demonstrate that the cardiac glycosides and aglycones can be quantitated in various mobile phase compositions of water and acetonitrile using pulsed amperometric detection.

3.2. Pulsed amperometric detection

The oxidation of the glycosides is believed to take place on the terminal digitoxose and lactone

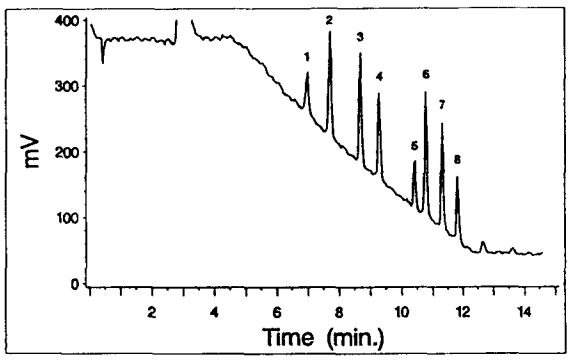


Fig. 2. Gradient separation of a mixed standard of digoxigenin (1), digoxigenin monodigitoxoside (2), digoxigenin bisdigitoxoside (3), digoxin (4), digitoxigenin (5), digitoxigenin monodigitoxoside (6), digitoxigenin bisdigitoxoside (7), and digitoxin (8). Conditions were indentical to those in Fig. 1 except that the mobile phase consisted of a linear gradient from water–ACN (90:10) to water–ACN (55:45) over 8 min at a flow-rate of 1.3 ml/min.

rings (Table 1). Both cyclic and acyclic conformations may be involved. Primary alcohols, polyhydroxyl compounds, glycols, and carbohydrates have been detected with PAD in alkaline media [19–23,25–28]. All of these are organic compounds with hydroxyl functional groups. Adsorption of the analyte onto the surface of the working electrode via hydrogen bonding with catalytic AuOH groups is important to several proposed detection mechanisms [22,29,30]. Hydroxyl groups on the terminal digitoxose and lactone rings are less sterically hindered than other functional groups elsewhere on the molecule. Thus for equal molar concentrations of terminal digitoxose and lactone units, the glycosides would be expected to have similar response factors. For the aglycones, the response factors would be expected to be different due to the absence of any digitoxose moieties. In addition to the lactone ring, oxidation may also take place on the hydroxyl group at the C-3 position. For example, the detection of cyclohexanol has been reported [22]. Other hydroxyl groups at positions C-12 and C-14 are more sterically hindered and would not be expected to be electroactive. For these reasons, both digitoxigenin and digoxigenin would be expected to have similar response factors.

The peak area vs. analyte mass data are presented graphically in Fig. 3. Each pair of aglycone, bisdigitoxoside, monodigitoxoside, and secondary glycoside from both cardenolide series behaved similarly. This was expected since the only structural difference between the digitoxin and digoxin cardenolide series is at the sterically hindered C-12 position of the aglycone (Table 1). The hydroxyl group present at the C-12 position on the digoxigenin aglycone is not electroactive, as evidenced by the similar behavior of digitoxigenin and digoxigenin. For the glycosides, the slopes of the curves are related to the molecular mass of the compound. Since, at a given mass, molar concentrations of the terminal digitoxose and lactone moieties are inversely proportional to the molecular mass (i.e. monodigitoxosides > bisdigitoxisides > secondary glycosides), the electrochemical response is greater for the lower-molecular-mass com-

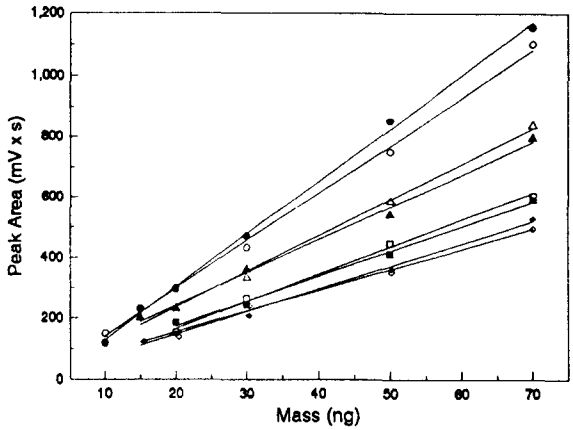


Fig. 3. Mean peak area vs. analyte mass for digitoxin, digoxin, and their metabolites. (■) digitoxin; (▲) digitoxigenin bisdigitoxoside; (●) digitoxigenin monodigitoxoside; (◆) digitoxigenin; (□) digoxin; (△) digoxigenin bisdigitoxoside; (○) digoxigenin monodigitoxoside; (◇) digoxigenin.

pounds. For the aglycones, the lack of any digitoxose moieties result in a lower oxidation current.

When the data in Fig. 3 was plotted as peak area vs. molar concentration of analyte, the resulting curves were found to be grouped into two distinct groups, the glycosides and the aglycones. At a given molarity, each glycoside has equal molar concentration of the terminal digitoxose and lactone units. As a result, they all have similar response factors. The lack of a digitoxose unit resulted in lower anodic oxidation rates for the aglycones. Upon closer inspection of the curves within the glycoside group, a pattern exists where the response factor is inversely related to the size of the compound: monodigitoxosides > bisdigitoxisides > secondary glycosides. Differences in response factors within the glycoside group may be attributed to small differences in molecular diffusion rates and oxidation reaction kinetics. Diffusion rates are inversely proportional to molecular mass according to Stokes' Law [31]. Larger molecules diffuse more slowly and would be less efficient at migrating to the surface of the gold working electrode. The kinetics of adsorption, dehydrogenation, electron and oxygen transfers, and displacement during the oxidation of the analyte may be also be influenced by the size of the molecule.

Table 2
Limits of detection for digitoxin, digoxin, and their metabolites

Compound	Limit of detection (injection volume, 10 μl)	
	Isocratic (ng/μl)	Gradient (ng/μl)
Digitoxin	1.43	0.40
Digitoxigenin bisdigitoxoside	1.22	0.28
Digitoxigenin monodigitoxoside	0.77	0.20
Digitoxigenin	1.34	0.35
Digoxin	1.06	0.23
Digoxigenin bisdigitoxoside	0.72	0.19
Digoxigenin monodigitoxoside	0.44	0.16
Digoxigenin	0.67	0.26

3.3. Limits of detection

The limits of detection for the various compounds are provided in Table 2 for both isocratic and gradient elutions. The conditions used are the same as those for Figs. 1 and 2. The limit of detection was calculated as the amount of compound which will produce a peak-height response equal to three times the detector baseline peak-to-peak noise. The limits of detection presented in Table 2 for PAD detection of the steroids are comparable to the limits of detection reported for UV detection at 220 nm [10,11,13,14]. However, PAD detection is more sensitive than the reported limits of detection for UV detection at 220 nm for the analysis of the digitoxose containing glycosides.

3.4. Response linearity

The curves in Fig. 3 are linear ($r^2 > 0.973$) in the range of 10–70 ng for the monodigitoxisides, 15–70 ng for the bisdigitoxosides and aglycones, and 20–70 ng for the secondary glycosides. The ranges were chosen to include concentrations typically used for chromatographic assays using UV detection [10–16]. The ranges are also applicable to pharmaceutical preparations of digitalis glycosides and extracts of *Digitalis*. The

method described here can be used for pharmaceutical quality control of digitoxin and digoxin tablets and injections, or for screening of *Digitalis* plant materials for maximum yield of the cardenolides. The mean coefficient of variation for the detector response in the range of 20–70 ng was 4.1% for the digitoxin cardenolide series and 7.6% for the digoxin cardenolide series.

3.5. Effect of ACN on detector response

The choice of organic modifier must be made with care. Some common oxygenated modifiers, such as methanol, acetone, dioxane, and tetrahydrofuran, produce prohibitively high background current. Acetonitrile is the most commonly used organic modifier for the reversed-phase separation of the cardiac glycosides. However, mobile phase compositions with high percentage of ACN greatly attenuated the peak areas of all of the cardiac glycosides and aglycones used in this study. Acetonitrile hinders detection by adsorbing to the surface of the gold working electrode [22,24]. The effect of % ACN composition on the peak area of a model compound, digoxigenin monodigitoxoside, is shown in Fig. 4. Digoxigenin monodigitoxoside was used because it is soluble in the mobile phase compositions chosen for this study. At ACN compositions of less than approximately 30%, detector response was not affected. At ACN compositions between 30% to 70%, the analyte peak area displays an inverse relationship with % ACN composition. At approximately 70% ACN, the baseline noise increased dramatically. At ACN compositions greater than 80%, the peak is no longer quantifiable due to decreased analyte response and increased background noise.

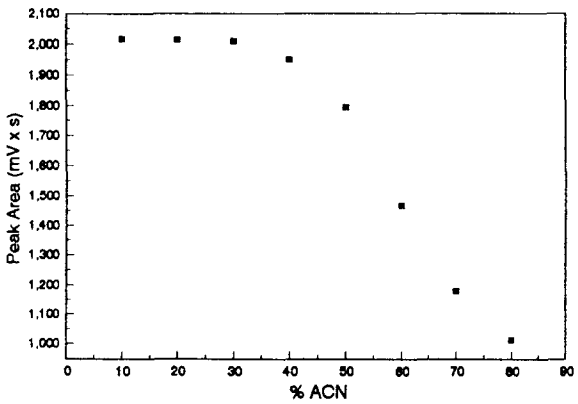


Fig. 4. Mean peak area vs. % mobile phase composition of ACN. Column was replaced with a zero dead volume connector. Solvent composition of the injected standard was the same as that of the mobile phase.

The isocratic separations of the digitoxin and digoxin cardenolide series were performed at the upper plateau region of the % ACN curve shown in Fig. 4. However, during the gradient separation shown in Fig. 2, peak areas of the compounds eluting at higher % ACN were probably attenuated. This explains why detection limits are higher for the digitoxin cardenolide series than for the digoxin cardenolide series during gradient elution.

3.6. Selectivity

Another major advantage of pulsed amperometric detection is direct and selective detection. No pre- or post-column derivatization is necessary. Selectivity is dependent on the oxidation potentials of the target and interfering compounds present in the sample. Adjustment of the applied potential (E_1) is made to reduce or eliminate interferences from other compounds. This helps reduce losses of the target analytes during clean-up of the sample extract or during derivatization reactions. In some cases, the adjustments of the % ACN composition in the mobile phase or the addition of ACN post-column can be made to reduce detector responses from potential interferences such as alcohols and simple sugars [22,24].

In conclusion, a HPLC method has been developed for the determination of the *Digitalis* cardiac glycosides with greater sensitivity and selectivity than UV detection at 220 nm. The analytes are separated on a conventional octadecylsilane column. Well-established HPLC methods can be easily modified to allow detection by PAD.

4. Safety

Safety precautions such as laboratory coats, gloves, safety goggles, and fume hoods should be used when handling solvents and cardioactive compounds.

Acknowledgements

The authors would like to thank Dionex Corporation for loaning the pulsed electrochemical detector. We are also grateful for the donation of digoxigenin monodigitoxoside from Burroughs Wellcome Company. The assistance of Paige Groninger for the preparation of the graphics is also greatly appreciated. Mention of commercial products is for identification only and does not constitute endorsement by the United States Department of Agriculture.

References

[1] K. Repke, in W. Wilbrandt (Editor), Proc. First Int. Pharmacol. Meeting, Vol. 3, Pergamon Press, New York, 1963, p. 47.
[2] G.T. Okita, in C. Fisch and B. Surawicz (Editors), Digitalis, Grune and Stratton, New York, 1969, p. 13.
[3] G.L. Lage and J.L. Spratt, J. Pharmacol. Exp. Ther., 152 (1966) 501.
[4] R.W. Jelliffe and D.H. Blakenhorn, J. Chromatogr., 12 (1963) 268.
[5] E. Watson, P. Tramell and S.M. Kalman, J. Chromatogr., 69 (1972) 157.
[6] L. Tan, J. Chromatogr., 45 (1969) 68.
[7] G.W. Ponder and J.T. Stewart, J. Chromatogr. A, 659 (1994) 177.
[8] S.M. Khafagy and A.N. Girgis, Planta Med., 25 (1974) 267.
[9] C.J. Clarke and P.H. Cobb, J. Chromatogr., 168 (1979) 541.
[10] W. Lindner and R.W. Frei, J. Chromatogr., 117 (1976) 81.
[11] M.C. Castle, J. Chromatogr., 115 (1975) 437.
[12] L. Sternson and R.D. Shaffer, J. Pharm. Sci., 67 (1978) 327.
[13] Y. Fujii, H. Fukuda, Y. Saito and M. Yamazaki, J. Chromatogr., 202 (1980) 139.
[14] F. Erni and R.W. Frei, J. Chromatogr., 130 (1977) 169.
[15] B. Pekic, S.M. Petrovic and B. Slavica, J. Chromatogr., 268 (1983) 237.
[16] B. Desta, E. Kwong and K.M. McErlane, J. Chromatogr., 240 (1982) 137.
[17] F. Nachtmann, H. Spitzy and R.W. Frei, J. Chromatogr., 122 (1976) 293.
[18] J.C. Gfeller, G. Frey and R.W. Frei, J. Chromatogr., 142 (1977) 271.
[19] S. Hughes and D.C. Johnson, Anal. Chim. Acta, 132 (1981) 11.
[20] R.D. Rocklin and C.A. Pohl, J. Liq. Chromatogr., 6 (1983) 1577.
[21] P. Edwards, Food Technol., 37 (1983) 53.
[22] D.C. Johnson and W.R. LaCourse, Anal. Chem., 62 (1990) 589A.
[23] B. Herbreteau, Analusis, 20 (1992) 355.
[24] W.R. LaCourse, W.A. Jackson and D.C. Johnson, Anal. Chem., 61 (1989) 2466.
[25] R.D. Rocklin and C.A. Pohl, J. Liq. Chromatogr., 30 (1983) 1577.
[26] P. Edwards and K.K. Haak, Amer. Lab., April (1983) 78.
[27] W.T. Edwards, C.A. Pohl and R. Rubin, Tappi J., 70 (1987) 138.
[28] J. Olechno, S.R. Carter, W.T. Edwards and D.G. Gillen, Am. Biotech. Lab., 5 (1987) 38.
[29] L.A. Larew and D.C. Johnson, J. Electroanal. Chem., 262 (1989) 167.
[30] Y.B. Vassilyev, O.A. Khazova and N.N. Nikolaeva, J. Electroanal. Chem., 196 (1985) 127.
[31] G.G. Stokes, Trans. Cambridge Phil. Soc., Ser. 9 (1851) No. 8.