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Development and validation of a liquid chromatographic–tandem mass spectrometric method for the determination of pibutidine in human urine

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Abstract

A liquid chromatographic–tandem mass spectrometric method for the rapid quantitative determination of pibutidine, an H_2-receptor antagonist, in human urine has been developed and validated over the concentration range 0.1–25.6 μg ml^{-1}. Urine samples were prepared based on a simple dilution with 0.05% acetic acid, followed by reversed-phase liquid chromatographic separation. Pibutidine and its internal standard ($^2H_{10}$-pibutidine) were ionized using an electrospray ionization interface and detected by tandem mass spectrometry in the selected reaction-monitoring mode. Completed validation demonstrated the method to be robust, accurate, precise and specific for the direct quantification of pibutidine in human urine. This method has enabled investigation of the urinary excretion of pibutidine following oral administration of pibutidine hydrochloride to healthy subjects.

Keywords: Pibutidine

1. Introduction

Pibutidine hydrochloride, 3-amino-4-{[(Z)-4-[[4-(piperidinomethyl)-2-pyridyl]oxy]-2-butenyl]amino}-3-cyclobutene-1,2-dione monohydrochloride or IT-066 (Fig. 1), is a potent H_2-receptor antagonist being developed for the treatment of peptic ulcer [1]. Previously, a novel method for characterization of pibutidine metabolites by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) demonstrated that pibutidine was the major component present in urine from volunteers receiving a single oral dose of pibutidine hydrochloride (40 mg); M-5, M-7, M-11 and M-12 were the major metabolites; and small quantities of M-1, M-6, M-8 and M-14 were also present [2]. It is known that the elimination of H_2-receptor antagonists from the body is mainly due to renal excretion, and that the renal function of patients affects the pharmacokinetics of H_2-antagonists [3]. As a subsequent part of the clinical investigations of pibutidine, it was necessary to develop a high-

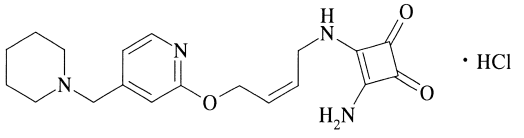

Fig. 1. Chemical structure of pibutidine hydrochloride.

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throughput method for the determination of pibutidine levels in urine to investigate the urinary excretion of pibutidine in humans.

The determination of plasma level of pibutidine was made with an electrochemical detector (ED) after separating pibutidine with a solid-phase extraction (SPE) procedure and high-performance liquid chromatography (HPLC) [4]. The ED can detect pibutidine with high sensitivity, but its specificity in identifying the pibutidine peak is low and detection of other coexisting substances can occur simultaneously with that of pibutidine. The LC–MS–MS technique has recently provided increased specificity without the need for tedious sample clean-up and is well-established for the routine determination of a variety of compounds [5–8]. In our previous study, the linear dynamic range of the ESI for pibutidine unfortunately appeared to be relatively narrow [2]. In order to overcome this limitation, in the present study, a deuterated analogue of pibutidine was used as an internal standard. The use of isotopically labeled internal standard in LC–MS quantitative analysis may also be needed to compensate for variations in instrumental and ionization conditions [9] and to eliminate the matrix effect [5,10].

This paper describes the development and validation of the LC–MS–MS method for determination of pibutidine in human urine, and the application of this method to the analysis of samples from healthy male volunteers in clinical study trials.

2. Experimental

2.1. Materials

Pibutidine hydrochloride and internal standard labeled with deuterium at the piperidine ring ($^2H_{10}$-pibutidine) were synthesized at Sumitomo Seika (Osaka, Japan) and Taisho (Saitama, Japan), respectively. Stock solutions of pibutidine and of $^2H_{10}$-pibutidine were prepared at 100 μg ml^{-1} as the hydrogen chloride (HCl) salt and at 10 μg ml^{-1} as the base in methanol, respectively. Both solutions were kept refrigerated (4°C) when not in use and were stable for several months. Authentic standards of the metabolites of pibutidine [2] were synthesized at Taisho. HPLC grade methanol, acetonitrile and analytical grade acetic acid were obtained from Wako (Osaka, Japan). HPLC grade distilled water was obtained from Kanto (Tokyo, Japan).

2.2. Instrumentation

The LC–MS–MS system consisted of a TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan ESI interface (Finnigan MAT, San Jose, CA, USA) and linked to an HP1050 LC system (Hewlett-Packard, Palo Alto, CA, USA). Chromatographic separations were performed using a Capcell PAK C_{18} UG80 S5 column (35×1.5 mm, Shiseido, Tokyo, Japan) at ambient temperature. The mobile phase was 0.05% acetic acid–acetonitrile (90:10, v/v) maintained at a flow-rate of 90 μl min^{-1}, and was degassed using helium. The autosampler was set to inject 10 μl of sample with a chromatographic run time of 4.4 min. An automated post-column switching valve (Valco, Houston, TX, USA) was employed to divert the eluent from the analytical column to waste for the first 1 min following injection and then into the MS–MS system for the rest of the run. ESI was performed in the positive ion mode with a spray voltage at +4.5 kV. The heated capillary temperature was maintained at 220°C. Nitrogen was generated by a Balston 75–760 nitrogen generation system (Whatman, Haverhill, MA, USA) and served both as sheath gas with an operating pressure of 85 p.s.i. and as auxiliary gas with a flow-rate of 10 units. The purity of the nitrogen was 99.8% or higher, depending on the flow-rate of the gas. Selected reaction monitoring (SRM) mode was used for the detection of pibutidine and $^2H_{10}$-pibutidine with a dwell time of 500 ms. The first quadrupole (Q_1) was set to transmit the protonated molecules at m/z 357 (pibutidine) and 367 ($^2H_{10}$-pibutidine). These ions were fragmented by collision-induced dissociation (CID) with argon (≈1.4 mTorr) at 25 eV in the second quadrupole (Q_2). Argon was ultra-high purity grade (99.999%, Taiyo Toyo Sanso, Osaka, Japan). The product ions at m/z 193 and 203 for pibutidine and $^2H_{10}$-pibutidine, respectively, were monitored via the third quadrupole (Q_3). Data were manipulated on Finnigan ICIS ver. 8.2.1 software.

2.3. Calibration standards, validation samples and quality control samples

Human drug-free urine was fortified with pibutidine at five concentrations of 0.1, 0.4, 1.6, 6.4 and 25.6 μg ml^{-1} as the HCl salt to obtain a set of calibration standards. Validation samples were prepared to cover the same concentration range as the calibration curve. Quality control (QC) samples were prepared at three concentrations of 0.20, 2.0 and 20 μg ml^{-1}.

2.4. Sample preparation

To a 10-μl aliquot of urine in a 2-ml centrifuge tube, 70 μl of stock solution of internal standard (0.7 μg $^2H_{10}$-pibutidine) and 1.2 ml of 0.05% acetic acid solution were added. The centrifuge tube was vortex-mixed for 30 s. A 10-μl portion of this injection sample was injected onto the LC–MS–MS system.

2.5. Assay validation

A calibration curve was constructed by plotting the peak area-ratio of the drug to internal standard versus drug concentration and using unweighted least-squares linear regression analysis. The precision and accuracy of the assay were determined by replicate analyses of validation samples and QC samples. The precision was evaluated as the relative standard deviation (RSD). The accuracy was expressed as the percentage of the theoretical concentration. Assay specificity for endogenous interfering peaks was assessed by running individual drug-free urine samples from six volunteers. The chromatographic retention time of authentic standards of the metabolites was also checked for interference. In addition, interference by coeluting metabolites was assessed quantitatively by analyzing duplicate urine samples containing pibutidine at 0.20, 2.0 and 20 μg ml^{-1}, which had been further fortified by the individual addition of possible metabolites (M-2, M-5 and M-12) at the same concentration as pibutidine. The ion suppression by matrix materials was evaluated at three concentration levels in six replicates by comparing the response of the QC samples to that of aqueous standards injected at the same level, which was determined in the same run. The freeze–thaw stability of pibutidine in urine was determined by analyzing the QC samples in duplicate after each freeze–thaw cycle in four analytical runs. The stability of pibutidine in the injection sample at room temperature, during storage in the autosampler, was determined by reanalyzing the same injection samples at 24 h after preparation with a freshly prepared calibration curve on the day of analysis. The long-term storage stability of pibutidine in human urine was checked by analyzing the QC samples that had been stored at −20°C for a period of time.

3. Results and discussion

3.1. ESI-MS–MS of pibutidine and $^2H_{10}$-pibutidine

Electrospray analysis of pibutidine and $^2H_{10}$-pibutidine revealed intense protonated molecules at m/z 357 and 367, respectively. These ions (precursor ions) were fragmented by CID in the Q_2 region of MS–MS to generate product ions (Fig. 2A and 2B). The product ions of pibutidine and $^2H_{10}$-pibutidine at m/z 193 and 203, respectively, were monitored by SRM. The formations of these product ions are believed to be caused by the cleavage of the C–O bond in the butenyloxy moiety (Fig. 2C). Under the operating conditions used, no difference between pibutidine and $^2H_{10}$-pibutidine was found in the efficiency of either ESI or CID.

3.2. Chromatographic separation

The detection of pibutidine and $^2H_{10}$-pibutidine by SRM turned out to be highly selective, and no interfering components whatsoever were observed in the chromatograms of urine samples. Therefore, no full chromatographic separation was necessary, and a retention time as short as only 2 min was found to be more than sufficient. This was accomplished by using a short (35-mm) HPLC column, which is convenient for running a high throughput of samples.

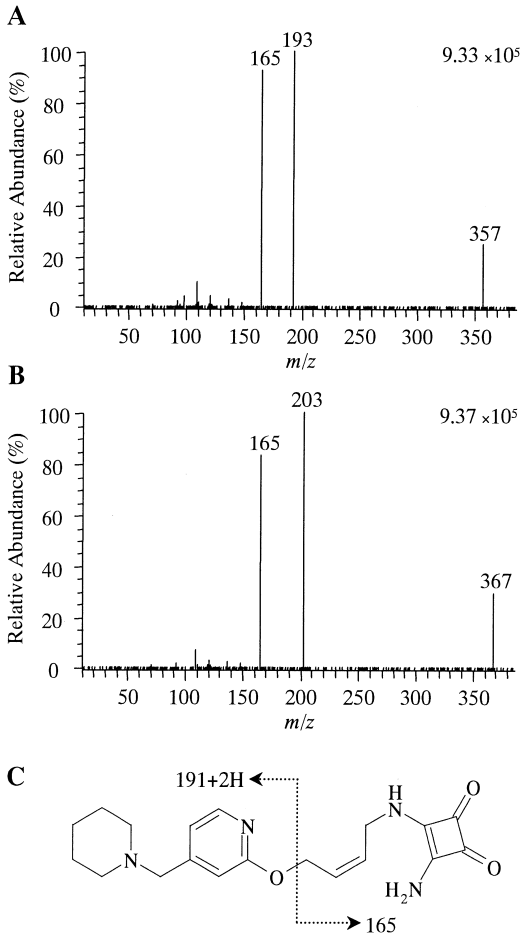

Fig. 2. Product ion spectra of the protonated molecules of (A) pibutidine and (B) $^{2}H_{10}$-pibutidine, and (C) proposed fragmentation pattern of pibutidine.

3.3. Linear dynamic range of ESI

In a previously reported study [2], the linear dynamic range of ESI for pibutidine appeared to be smaller with use of external standardization. The relationship between the peak area and injected amount of the analyte was nonlinear and was fitted by quadratic regression analysis, when the injected amount range was not less than two orders of magnitude (10–1000 pg, Fig. 3A). Since the linear dynamic range of ESI is very much dependent on the analyte and mobile phase [11,12], modification of the mobile phase [13] is one strategy for overcoming this limitation, but is not necessarily easy. Therefore, we used internal standardization with an isotopically labeled analogue, as a result of which the calibration curve of the peak area ratio versus injected amount was well fitted even with unweighted linear regression analysis (Fig. 3B).

3.4. Assay validation

3.4.1. Calibration curve

The lower limit of quantification (LOQ) was 0.1 μg ml^{-1} using 10 μl of urine (~7 pg as injected amount of pibutidine). Typical chromatograms of calibration standards at 0.1 and 6.4 μg ml^{-1} are shown in Fig. 4B and C. The relationship between the peak area ratio of pibutidine to internal standard and the concentration of pibutidine in human urine was linear over a concentration range greater than two orders of magnitude (0.1–25.6 μg ml^{-1} as the HCl salt). For six daily runs, all the calibration lines passed near the origin, and the mean and RSD of their slopes were 0.014785 and 1.2%, respectively. All of the regression coefficients were greater than 0.999.

3.4.2. Precision and accuracy of the assay

The intra-day assay variability was determined by analyzing validation samples containing pibutidine at five levels utilized for constructing calibration curves. Results are summarized in Table 1. The intra-day precision defined as RSD was less than 7% at all concentrations over the range of 0.1–25.6 μg ml^{-1}. The intra-day accuracy defined as the percentage of the theoretical concentration was within 100±2%. The inter-day variability of the assay method, assessed by replicate analysis of QC samples at three concentrations on 5 separate days, is presented in Table 2. The inter-day precision and accuracy were less than 7% and within 100±3%, respectively, over the entire tested concentration range.

3.4.3. Specificity

Individual control human urine obtained from six volunteers was found to contain no endogenous compounds that could interfere with the assay. A representative chromatogram is shown in Fig. 4A. Most urinary metabolites of pibutidine [2] were found to elute at different retention times than

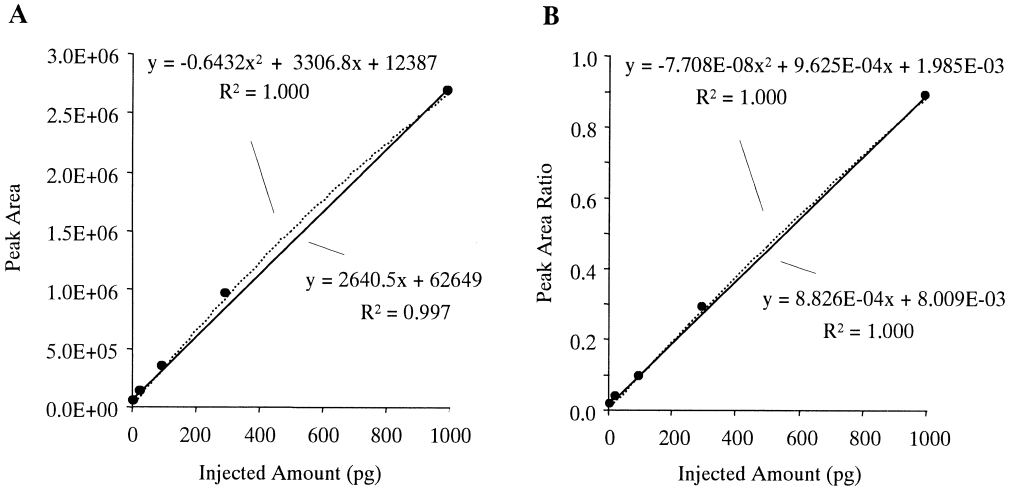

Fig. 3. Calibration curves of pibutidine by (A) external and (B) internal standard methods.

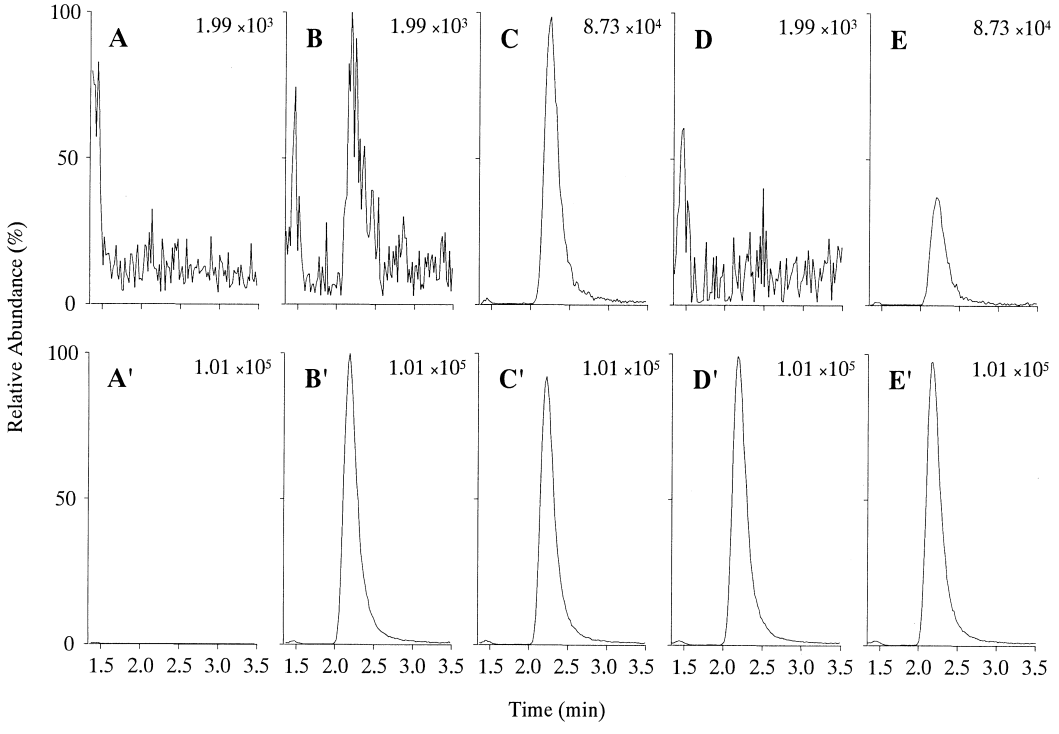

Fig. 4. Representative LC–MS–MS chromatograms of urine samples obtained by selected reaction monitoring at m/z 357→193 for pibutidine (upper) and m/z 367→203 for internal standard (lower); (A and A′) blank urine; (B and B′) blank urine spiked with pibutidine hydrochloride at 0.1 μg ml^{-1}; (C and C′) blank urine spiked with pibutidine hydrochloride at 6.4 μg ml^{-1}; (D and D′) pre-dose urine; (E and E′) urine collected 0–24 h after a 20-mg dose of pibutidine hydrochloride (assayed level 2.35 μg ml^{-1}). The numbers in the upper right-hand corner of the chromatograms indicate the peak height expressed in arbitrary units.

Table 1
Intra-day variability for the assay of pibutidine in urine

Nominal concentration (μg/ml)	Observed concentration[a] (μg/ml)	RSD (%)	Percentage of theoretical value
0.1001	0.1017	6.3	101.6
0.4004	0.4006	3.3	100.1
1.602	1.599	2.2	99.8
6.406	6.362	1.2	99.3
25.63	25.91	1.5	101.1

[a] Mean of six analyses at each level.

pibutidine (2.0 min), with the exception of M-2 (2.2 min) and M-5 (1.7 min). In addition, it was expected that M-12 (45.1 min) could coelute with pibutidine by carry-over ten samples later. When the influence of these metabolites was quantitatively investigated, no interference was encountered: the concentrations found ranged from 97.3 to 101.2%, 94.7 to 107.4% and 93.2 to 104.7% of the theoretical concentrations of pibutidine for coelutions with M-2, M-5 and M-12, respectively.

3.4.4. Matrix effect

Careful assessment of matrix effects should be an integral and important part of validation of any quantitative LC–MS–MS method utilized to support a long-term clinical pharmacokinetic program [14]. The peak areas of the analyte in urine samples were 78.2, 70.0 and 74.2% at 0.20, 2.0 and 20 μg ml^{-1}, respectively, compared with those for the corresponding aqueous standards. On the other hand, the peak area ratios of the analyte to internal standard in urine samples were respectively 104.8, 93.3 and 95.3% of those of the aqueous standards, because there was no difference between the drug and the internal standard in the signal suppression by matrix materials. It was demonstrated that matrix effects were eliminated or minimized by the use of the deuterated analogue as internal standard.

Table 2
Inter-day variability for the assay of pibutidine in urine

Nominal concentration (μg/ml)	Observed concentration[a] (μg/ml)	RSD (%)	Percentage of theoretical value
0.2002	0.1955	6.1	97.6
2.002	1.968	2.5	98.3
20.02	19.63	2.2	98.1

[a] Based on five runs, with duplicates at each level in each run.

3.4.5. Stability studies

The results of the freeze–thaw stability studies are shown in Table 3. Quantification of the analyte in urine subjected to a number of freeze–thaw (−20°C to room temperature) cycles showed that no stability problem for the analyte occurred after four cycles, indicating that the urine samples can be frozen and thawed at least four times prior to analyses. Another study, which was performed to evaluate the stability of the injection samples in the autosampler tray, showed that no degradation of the analyte had taken place over a 24-h storage period: the final concentrations of pibutidine ranged from 94.5 to 105.6% of the theoretical values. In addition, the long-term stability of pibutidine in QC samples after eight months of storage at −20°C was also evaluated. The concentrations found ranged from 95.1 to 101.9% of the theoretical values. It was thus demonstrated that pibutidine was stable in human urine for at least 8 months at −20°C.

3.5. Clinical application

This method has been successfully applied to the determination of pibutidine in urine from subjects following 5- to 80-mg oral doses of pibutidine hydrochloride [4]. Representative chromatograms of urine samples collected prior to administration and over a 24-h period after a 20-mg oral dose are shown in Fig. 4D and E, respectively. In each clinical sample run, a set of QC samples was assayed in duplicate at three levels concurrently with the cali-

Table 3
Freeze–thaw stability of pibutidine in human urine

Nominal concentration (μg/ml)	Percentage of theoretical value				
	Pre	Cycle no.			
		1	2	3	4
0.2002	98.1	107.5	98.3	100.5	86.7
0.2002	104.2	98.0	92.8	107.5	98.7
2.002	97.6	97.0	105.4	93.6	96.8
2.002	100.7	94.5	105.1	93.3	97.8
20.02	94.2	93.2	100.2	97.1	97.6
20.02	102.1	97.1	105.0	98.3	96.8

bration standards. The correlation coefficients and the accuracy for each of the QC samples were greater than 0.999 and within 100±6%, respectively, in all of the assay runs.

4. Conclusions

A method for the direct determination of pibutidine in human urine was developed and validated using tandem mass spectrometric methodology. It was demonstrated that use of an isotopically labeled internal standard in quantitative LC–ESI-MS analyses yields a much more efficient technique with linearity of the calibration curve and elimination of the influence of coeluting endogenous substances and drug metabolites on detector response of analyte. Validation experiments showed that the assay has very good precision and accuracy over a wide concentration range. In addition, the short run time (<5 min) allowed a high throughput assaying of samples. The present method has proven to be suitable for analysis of a large number of urine samples in clinical study trials.

References

[1] Y. Isobe, H. Kijima, C. Ito, H. Nagai, M. Muramatsu, I. Arai, K. Fukushima, Jpn. Pharmacol. Ther. 27 (1999) 261–271.
[2] K. Kato, S. Jingu, N. Ogawa, S. Higuchi, Rapid Commun. Mass Spectrom. 13 (1999) 1626–1632.
[3] J.H. Lin, Clin. Pharmacokinet. 20 (1991) 218–236.
[4] J. Azuma, T. Aoki, Y. Seto, Jpn. Phamacol. Ther. 27 (1999) s973–s990.
[5] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pham. Biomed. Anal. 18 (1998) 347–357.
[6] T.A. Neubecker, M.A. Coombs, M. Quijano, T.P. O'Neill, C.A. Cruze, R.L.M. Dobson, J. Chromatogr. B 718 (1998) 225–233.
[7] G.J. Dear, I.J. Fraser, D.K. Patel, J. Long, S. Pleasance, J. Chromatogr. A 794 (1998) 27–36.
[8] R. Burton, M. Mummert, J. Newton, R. Brouard, D. Wu, J. Pharm. Biomed. Anal. 15 (1997) 1913–1922.
[9] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. B 692 (1997) 7–14.
[10] B.K. Matuszewski, C.M. Chavez-Eng, M.L. Constanzer, J. Chromatogr. B 716 (1998) 195–208.
[11] A. Raffaelli, A.P. Bruins, Rapid Commun. Mass Spectrom. 5 (1991) 269–275.
[12] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972A–986A.
[13] M. Stefansson, P.J.R. Sjöberg, K.E. Markides, Anal. Chem. 68 (1996) 1792–1797.
[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.