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Validated HPLC method for the determination of gabapentin in human plasma using pre-column derivatization with 1-fluoro-2,4-dinitrobenzene and its application to a pharmacokinetic study

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Abstract

A rapid, sensitive and accurate high-performance liquid chromatographic method with UV detection was developed and validated for the quantification of gabapentin in human plasma. Gabapentin was quantified using pre-column derivatization with 1-fluoro-2,4-dinitrobenzene following protein precipitation of plasma with acetonitrile. Amlodipine was used as internal standard. The chromatographic separation was carried out on a Nova-Pak C_{18} column using a mixture of 50 mM NaH_2PO_4 (pH = 2.5)–acetonitrile (30:70, v/v) as mobile phase with UV detection at 360 nm. The flow rate was set at 1.5 ml/min. The method was linear over the range of 0.05–5 μg/ml of gabapentin in plasma ($r^2 > 0.999$). The within-day and between-day precision values were in the range of 2–5%. The limit of quantification of the method was 0.05 μg/ml. The method was successfully used to study the pharmacokinetics of gabapentin in healthy volunteers.

Keywords: Gabapentin; Epilepsy; 1-Fluoro-2,4-dinitrobenzene; HPLC; UV-detection

1. Introduction

Gabapentin, 1-(aminomethyl) cyclohexane acetic acid (Fig. 1), is a structural analogue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and a potent antiepileptic drug. Gabapentin is used for the treatment of complex partial seizures, with or without secondary generalization in patients over 12 years of age [1]. Gabapentin action is attributed to the irreversible inhibition of the GABA-transaminase enzyme and preventing the degradation of GABA in the brain [2]. Also, it has been shown that the action of gabapentin is possibly due to its high binding to α_2-δ protein, an auxiliary subunit of voltage-gated calcium channels. Potent binding at this site reduces the synaptic release of several neurotransmitters and reduce neuronal excitability and seizures [3]. Gabapentin is rapidly absorbed after oral administration, and reaches maximal plasma concentrations after 2–3 h. Gabapentin is not metabolized and entirely excreted unchanged in the urine. The drug does not bind to plasma proteins [4].

A reliable and simple method of determination is necessary for clinical and pharmacokinetics study of drugs. Gabapentin has no significant ultraviolet, visible or fluorescence absorption. Several HPLC methods have been published for determination of gabapentin in human plasma using different derivatizing reagents such as 2,4,6-trinitrobenzene sulphonic acid [5,6], phenylisothiocyanate (PITC) [7], *O*-phthaldialdehyde (OPA) [8–14], 4-chloro-7-nitrobenzofurazan [15], 9-fluorenylmethylchloroformate [16] and 1, 2-naphthoquinone-4-sulphonic acid sodium salt [17].

In the present study, a simple HPLC method with UV detection is described for the determination of gabapentin in human plasma. In this method 1-fluoro-2,4-dinitrobenzene (FDNB) (Fig. 1), the color labeling reagent for primary and secondary amines, has been used for derivatization of gabapentin for the first time. Recently, FDNB has been used for HPLC determination of vertilmicin in rat serum [18] and amikacin in the skin [19].

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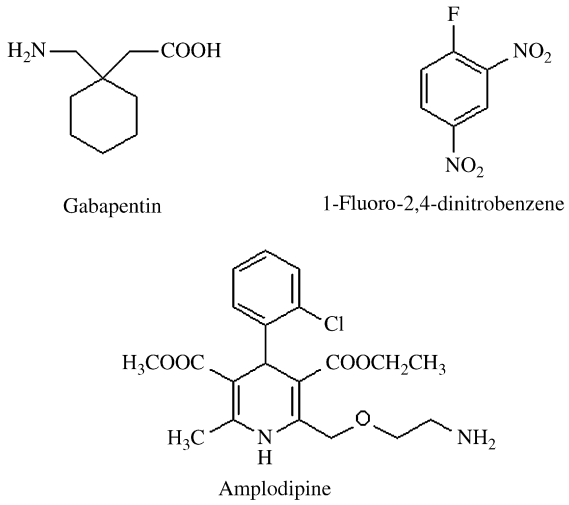

Fig. 1. Chemical structure of gabapentin, 1-fluoro-2,4-dinitrobenzene and amlodipine (I.S.).

2. Experimental

2.1. Chemicals

Gabapentin was from Ranbaxy (India) and kindly provided by Daru-Darman Pars Company (Tehran, Iran). FDNB was purchased from Fluka (Switzerland). Amlodipine besylate (internal standard, I.S.) was from Eczacibasi (Istanbul, Turkey).

Acetonitrile was HPLC grade and purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and used without any further purification. Distilled water was purified by a Millipore System MilliQ.

2.2. Instrumentation

The HPLC system consisted of a 600 Pump, 710 plus Autosampler and a variable 480 UV Detector all from Waters (Milford, MA, USA). The data processing system was a multichannel Chrom & Spec software for chromatography, version 1.5 x.

2.3. Standard solutions

Stock standard solution of gabapentin was prepared by dissolving an appropriate amount of the compound in distilled water to give a final concentration of 100 μg/ml. Standard solutions of gabapentin (0.5, 1, 2, 5, 10, 20, 40, 50 μg/ml) were prepared by subsequent dilution. Fifty microliters of standard solutions were used to spike 0.5 ml plasma samples for calibration curves to reach to concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 5 μg/ml of gabapentin. A solution of I.S. was prepared by dissolving amlodipine besylate in water to a final concentration of 30 μg/ml. All these solutions were stored at 4 °C.

Stock solution of FDNB (0.06 M) was prepared by dissolving 1.14 g of reagent in 100 ml of acetonitrile. This reagent should be handled carefully since it is a skin irritant.

A 0.25 M borate buffer containing 0.25 M KCl was prepared by dissolving appropriate amounts of H_3BO_3 and KCl in water and adjusting the pH to 8.2 by adding 2 N NaOH.

2.4. Sample preparation

To 500 μl of volunteer plasma sample in a test tube, 50 μl of I.S. solution (30 μg/ml) were added and vortex-mixed for 5 s. The samples were deproteinized using 600 μl of acetonitrile. The test tubes were vortex-mixed and centrifuged (1500 × *g* for 7 min). To 500 μl of the supernatant in a clean test tube, 200 μl borate buffer, 30 μl FDNB reagent and 1.2 ml acetonitrile were added. The mixture was vortex-mixed for 5 s and kept in dark at 65 °C for 10 min. After cooling to room temperature, 50 μl of 1 M HCl solution was added and 50 μl injected into HPLC system.

2.5. Chromatographic conditions

Separation was achieved using a Nova-Pak® C_{18} 4 μm column (250 mm × 4.6 mm, Waters, Milford, MA, USA). The isocratic mobile phase, pumped at a flow rate of 1.5 ml/min, consisted of 50 mM NaH_2PO_4 (pH = 2.5)–acetonitrile (30:70, v/v) prepared daily, degassed by passing through a 0.45 μm filter and ultrasonicated for 10 min. All separations were performed at room temperature. Detection was performed at 360 nm.

2.6. Optimization of reaction conditions

The derivatization of gabapentin with FDNB was optimized using solutions of 5 μg/ml of the drug in the presence of I.S. To optimize the reaction condition, various amounts of FDNB reagent ranging from 10 to 60 μl were studied at two different temperatures (65° and 90 °C) and different reaction times. In optimized condition, 30 μl of reagent was used and the reaction mixture was kept at 65 °C for 10 min.

2.7. Calibration

Six series of calibration solutions were prepared by spiking 50 μl of gabapentin standard solutions and 50 μl of I.S. in 500 μl of blank human plasma to give final concentrations over the range of 0.05–5 μg/ml.

Sample preparation and HPLC analysis were performed as described above. Calibration curves were constructed by plotting the measured peak area ratios of gabapentin to I.S. versus concentrations of standard samples and statistical analysis was performed.

2.8. Precision and accuracy

In order to evaluate the within-day and between-day precision and accuracy, six replicates of standard plasma solutions at three different concentrations (0.05, 1 and 5 μg/ml) were assayed on one day and three separate days.

2.9. Absolute recovery

An aliquot of 50 μl of gabapentin solution (50 μg/ml), I.S. and 500 μl of blank plasma were added to six test tubes. The samples were processed as described in sample preparation method. Absolute recovery was calculated by comparing the peak areas obtained with samples achieved by derivatization of aqueous solutions.

2.10. Pharmacokinetic study

The developed method was applied to investigate the pharmacokinetics of gabapentin in human after administration of a single oral dose of Neurontin 400 mg (Parke-Davis, Germany, Lot No: 0076125). Twelve healthy adult male volunteers (age: 28.3 ± 3.4 years, height: 173.5 ± 6.4 cm, body mass: 77.4 ± 4.4 kg) selected and participated in the study based on acceptable physical examination, medical history and clinical laboratory test results. All subjects gave written consent to their participation after having been informed verbally by the medical supervisor about the experimental procedures. After an overnight fast, each subject received a single 400 mg oral dose of gabapentin. Blood samples (5 ml) were drawn into heparinized test tubes immediately before (0) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 h following drug administration. Blood samples were centrifuged at 2000 × *g* for 10 min and plasma samples were separated and stored at −20 °C until analysis. Before analysis, the plasma samples were thawed at 18 °C.

3. Results

3.1. Derivatization reaction

Reaction of FDNB with primary amines is carried out in the presence of borate buffer (pH = 8.2). A hydrolysis product of FDNB (dinitrophenolate) is also formed in the reaction mixture which turns to dinitrophenol after acidification at the end of derivatization [20]. The UV absorption spectra of the gabapentin–FDNB derivative showed a maximum at 360 nm which was used as the HPLC analytical wavelength.

Using different amounts of the reagent showed that by increasing the amount of the reagent the peak area of the gabapentin derivative was increased. The completion of the reaction was achieved using 30 μl of the reagent (Fig. 2). Larger amounts of reagent did not show any effect on reaction yield but increased proportion of interfering by-products.

Using different reaction temperatures, it was found that the reaction was completed after 10 min at 65 °C or 5 min at 90 °C (Fig. 2). The reaction product was not stable at 90 °C but, at lower temperature, it was proceeded slowly and remained stable. No reaction was achieved at room temperature. The best results were obtained at 65 °C within 10 min.

3.2. Chromatography

Different mobile systems were used for separation of gabapentin–FDNB derivative using Nova-Pak C_{18} column. The best results was achieved using a mixture of 50 mM NaH_2PO_4 (pH = 2.5)–acetonitrile (30:70, v/v). Excellent chromatographic specificity with no endogenous plasma interference was obtained. Several compounds including baclofen, azithromycin and amlodipine were tested and amlodipine was chosen as an internal standard with an appropriate retention time and consistency during derivatization. Typical chromatograms obtained from blank plasma, plasma sample spiked with gabapentin and plasma sample from a volunteer after a single oral dose of 400 mg gabapentin are presented in Fig. 3. Under described chromatographic conditions, gabapentin and I.S. were well resolved in plasma samples and eluted at about 3.5 and 6 min, respectively. No interfering peaks of endogenous plasma components or derivatizing reagent were found at the retention time of gabapentin or internal standard in blank plasma.

3.3. Absolute recovery and analyte stability

The mean recoveries including the derivatization step were 100%, 96% and 97% at 0.1, 1 and 5 μg/ml, respectively, and

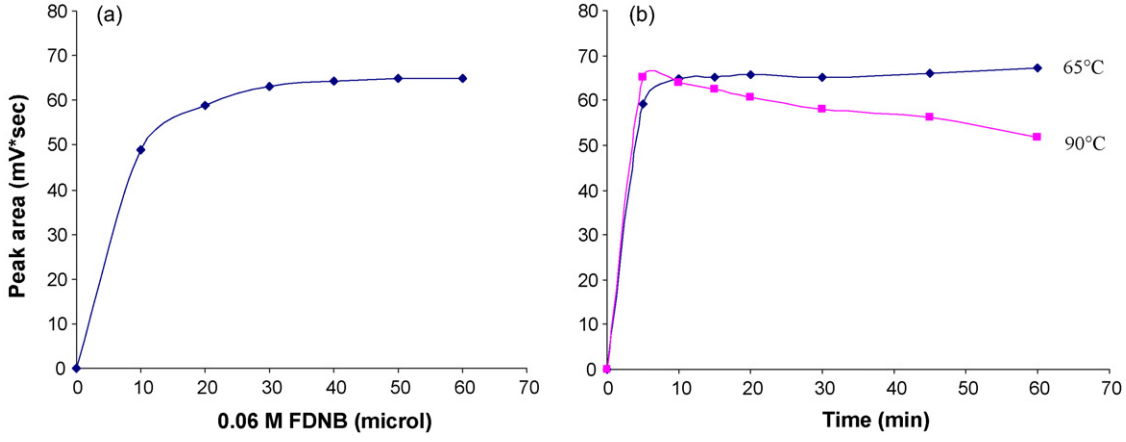

Fig. 2. Effects of reagent amount (65 °C for 10 min) (a) and time and temperature (b) on the peak areas of the FDNB-gabapentin derivative ($n = 3$).

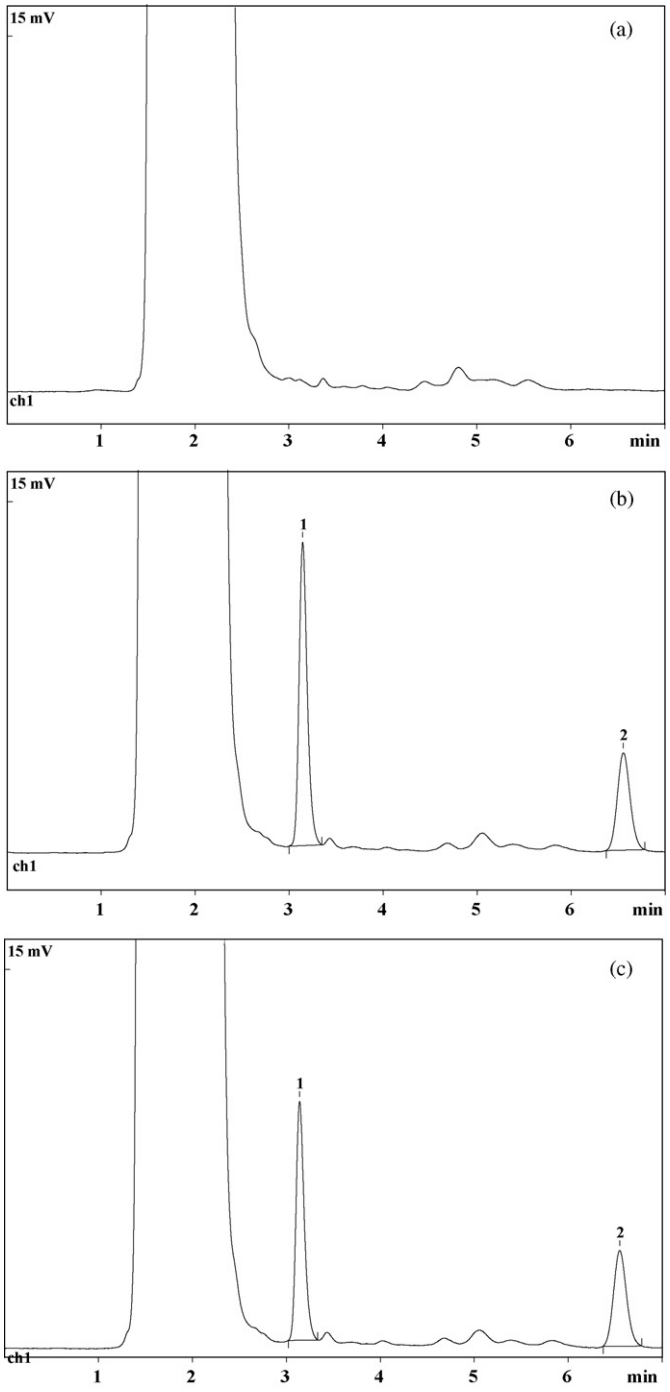

Fig. 3. HPLC chromatograms of gabapentin and I.S: (a) drug free plasma; (b) plasma sample spiked with gabapentin (4 μg/ml) and I.S.; (c) human plasma sample, 6 h after oral administration of 400 mg gabapentin. Peaks: 1, gabapentin derivative; 2, I.S. derivative.

was calculated by comparing peak areas of gabapentin derivative in spiked plasma samples and standard solutions. The absolute recovery of the I.S. was about 100%.

Gabapentin was found to be stable in human plasma for at least 2 weeks. The derivatized products of gabapentin and the internal standard were also shown to be stable for at least 24 h at room temperature.

Table 1
Statistical data of calibration curves of gabapentin in spiked plasma ($n = 6$)

Parameters	Gabapentin
Linearity	0.05–5 μg/ml
Regression equation	$Y = 0.784x + 0.125$
S.D. of slope	0.007
R.S.D. of slope (%)	0.89
S.D. of intercept	0.016
Correlation coefficient	0.9997

3.4. Linearity

Calibration curves were constructed using six series of plasma samples spiked at concentration levels in the range of 0.05–5 μg/ml. Linear relationship was obtained between the peak area ratio of gabapentin to that of the internal standard versus the corresponding concentration, as shown by the equation presented in Table 1. The linearity of the calibration curve is validated by the high value of the correlation coefficient.

3.5. Accuracy and precision

The accuracy and precision were determined using calibration standard curve prepared for gabapentin in the range of 0.05–5 μg/ml for each day. Within-day and between-day data given in Table 2, indicate CV values <4.6% and error <2%.

3.6. Sensitivity

The limit of quantification with CV < 4.6% was found to be 0.05 μg/ml for gabapentin. The reliable limit of detection with a S/N ratio of 3 was found to be 0.01 μg/ml.

3.7. Application to pharmacokinetic study

The average plasma concentration–time profile of gabapentin after administration of a single 400 mg neurontin tablet to 12 volunteers is shown in Fig. 4. The following pharmacokinetic parameters (mean ± S.D.) were provided for neurontin: $C_{max} = 3.33 \pm 1.19$ μg/ml, $AUC_{0-24} = 29.09 \pm 9.22$ μg h/ml, $T_{max} = 3.13 \pm 0.43$ h, elimination half-life = 6.81 ± 1.12 h.

Table 2
Precision and accuracy of method for determination of gabapentin in spiked plasma ($n = 18$; six sets for 3 days)

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Precision (%)	Error (%)
Within-day ($n = 6$)			
0.050	0.051 ± 0.002	3.70	2.00
1.000	0.995 ± 0.045	4.49	−0.50
5.000	4.982 ± 0.111	2.23	−0.36
Between- day ($n = 18$)			
0.050	0.051 ± 0.002	6.12	−2.00
1.000	0.997 ± 0.48	4.43	−0.30
5.000	5.024 ± 0.153	3.04	0.48

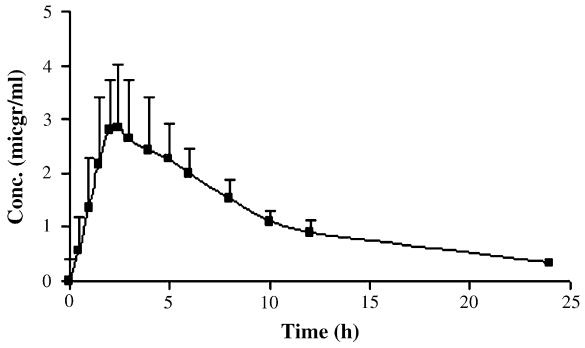

Fig. 4. Concentration–time profile of gabapentin after oral administration of a single dose 400 mg neurontin to 12 volunteers.

The observed values of pharmacokinetic parameters were comparable to those reported in previous studies [15].

4. Discussion

Gabapentin shows weak absorption in UV range and cannot be detected by UV detectors. Derivatization by chromophoric reagents increases the sensitivity of gabapentin detection. Different derivatizing reagents have been used before for determination of gabapentin [5–17].

In most published methods, *O*-phthaldialdehyde was used [8–14]. The fluorescent OPA-drug derivative should be injected immediately after preparation because of the instability of the adduct. The method is difficult to apply for pharmacokinetic studies if automated instrumentation is not available. Derivatization with phenylisothiocyanate is simple but this reagent degrades in contact with water and the extraction residue from plasma should be completely free from water before the addition of reagent [7]. Using 4-chloro-7-nitrobenzofurazan as labeling reagent, the adduct should be extracted and detected by fluorimetry [15]. The reaction time for gabapentin and 9-fluorenyl methyl chloroformate is about 10 min but the column temperature of 60 °C is needed for separation of the adduct [16]. In the method reported by Sagirli et al. [17], solid-phase extraction, derivatization with 1, 2-naphthoquinone-4-sulphonic acid and extraction of the reaction product were needed and the total run time of HPLC method was 15 min.

The present method using FDNB provides stable derivatives with absorption maximum at 360 nm. Strong absorbance at 360 nm, leads to enough sensitivity for the analysis. The developed isocratic HPLC conditions allowed separation of gabapentin and I.S. from endogenous and reagent products within 7 min.

Although several methods are now available for determination of gabapentin in human plasma, this is the first report of the HPLC analysis of gabapentin with UV detection using FDNB as derivatizing reagent without the limitations of the previously reported methods.

Compared with the other reported methods, the proposed method is more simple, as it is conducted in 10 min at 65 °C. Although the reaction time is 10 min, it can be applied in a great number of samples simultaneously, thus the total analysis time is relatively short. The sample preparation is very short and simple and no extraction of gabapentin or reaction product is needed. Finally, in comparison with other methods previously reported for HPLC determination of gabapentin in plasma, the developed method is simple, rapid, accurate, sensitive and comparable to HPLC methods with fluorimetric detection. The reported method is suitable for processing of multiple samples in a limited amount of time for pharmacokinetic studies.

References

[1] K.B. Tallian, M.C. Nahata, W. Lo, C.Y. Tsao, J. Clin. Pharm. Ther. 29 (2004) 511.
[2] D. Ouellet, H.N. Bockbrader, D.L. Wesche, D.Y. Shapiro, E. Garofalo, Epilepsy Res. 47 (2001) 229.
[3] C.P. Talor, T. Angelotti, E. Fauman, Epilepsy Res. 73 (2007) 137.
[4] B.E. Gidal, L.L. Radulovic, S. Kruger, P. Rutecki, M. Pitterle, H.N. Bockbrader, Epilepsy Res. 40 (2000) 123.
[5] H. Hengy, E.U. Kolle, J. Chromatogr. 341 (1985) 473.
[6] J.M. Juenke, P.I. Brown, G.A. Mc Millin, F.M. Urry, Clin. Chem. 49 (2003) 1198.
[7] Z. Zhu, L. Neirinck, J. Chromatogr. B 779 (2002) 307.
[8] G. Forrest, G.J. Sills, J.P. Leach, M.J. Brodie, J. Chromatogr. B 681 (1996) 421.
[9] H. Ratnaraj, P.N. Patsalos, Ther. Drug Monit. 20 (1998) 430.
[10] Q. Jiang, S. Li, J. Chromatogr. B 727 (1999) 119.
[11] P.H. Tang, M.V. Miles, T.A. Glauser, T. De Grauw, J. Chromatogr. B 727 (1999) 125.
[12] D.F. Chollet, L. Goumaz, C. Juliano, G. Anderegg, J. Chromatogr. B 746 (2000) 311.
[13] J.D. Gauthier, R. Gupta, Clin. Chem. 48 (2002) 2259.
[14] T.A.C. Vermeij, P.M. Edelbroek, J. Chromatogr. B 810 (2004) 297.
[15] Gh. Bahrami, B. Mohammadi, J. Chromatogr. B 837 (2006) 24.
[16] Gh. Bahrami, A. Kiani, J. Chromatogr. B 835 (2006) 123.
[17] O. Sagirli, S.M. Cetin, A. Oral, J. Pharm. Biomed. Anal. 42 (2006) 618.
[18] M. Zhou, G. Wei, Y. Liu, Y. sun, Sh. Xiao, L. Lu, Ch. Liu, D. Zhong, J. Chromatogr. B 798 (2003) 43.
[19] S. Nicoli, P. Santi, J. Pharm. Biomed. Anal. 41 (2006) 994.
[20] G. Paraskevas, J. Atta-Politou, M. Koupparis, J. Pharm. Biomed. Anal. 29 (2002) 865.