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Hollow-fiber micro-extraction combined with HPLC for the determination of sitagliptin in urine samples

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Abstract: In this study, a three-phase, hollow-fiber, liquid-phase micro-extraction (HF–LPME) method coupled with high performance liquid chromatography was successfully developed for the determination of trace levels of the anti-diabetic drug sitagliptin (STG) in urine samples. Sitagliptin was extracted from 15 mL of the basic sample solution with a pH of 8.5 into an organic extracting solvent (*n*-octanol) impregnated in the pores of a hollow fiber and then back extracted into an acidified aqueous solution in the lumen of the hollow fiber with a pH of 3. After extraction, 20 μL of the acceptor phase was injected into HPLC system. In order to obtain high extraction efficiency, the parameters affecting the HF–LPME, including pH of the source and receiving phases, type of organic phase, ionic strength, stirring rate, extraction time, the volume ratio of donor phase to acceptor phase and temperature, were studied and optimized. Under the optimized conditions, enrichment factors up to 88 were achieved and the relative standard deviation of the method was in the range from 3 to 6 %. The results indicated that the HF–LPME method has an excellent clean-up capacity and a high pre-concentration factor and could serve as a simple and sensitive method for monitoring the drug in urine samples.

Keywords: anti-diabetic drug; drug analysis; chromatography; biological sample; pre-concentration.

INTRODUCTION

Type 2 diabetes mellitus is a common chronic condition that causes significant morbidity and mortality worldwide and is a growing global public health concern.

Sitagliptin (STG), (*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, is an orally active, potent and selective inhibitor of dipeptidylpeptidase-4 (DPP-4 inhibitor), for the treatment of type 2 diabetes.[1,2] STG was the first DPP-4 inhibitor to gain

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the approval of the Food and Drug Administration (FDA) in October 2006 for both monotherapy and in combination with other anti-diabetic agents for the treatment of type 2 diabetes mellitus.[3] Following oral administration of a 100-mg tablet, the absolute bioavailability is approximately 87 % and therapeutic concentration (c_{max}) is 950 nmol L^{-1} in healthy individuals. Eighty seven percent of STG is eliminated *via* renal excretion (79 % unchanged) with a renal clearance in the region of 350 mL min^{-1}.[1,2,4]

Several liquid chromatographic methods with ultraviolet and mass spectrometric detection have been reported in the literature for the quantitative determination of STG in biological fluids. Techniques utilizing HPLC alone do not have an adequate lower limit of quantification (*LLOQ*) suitable for monitoring sitagliptin in low concentrations.[5] Recent LC/MS/MS methods showed improvement in sensitivity, however, a mass spectrometer is very expensive.[6,7] Therefore, due to the low concentration of sitagliptin in biological samples, a pretreatment and pre-concentration step is generally required prior to determination of trace amounts of the drug. Various sample preparation procedures have been applied for STG in biological fluids, such as liquid–liquid extraction (LLE) and solid phase extraction (SPE).[5,8] The liquid micro extraction method is a relatively new method for sample preparation. This method has some advantages over conventional extraction methods such as LLE and SPE.[9–11] In 1999, Pedersen-Bjergaard and Rasmussen suggested using porous hollow polypropylene fibers as a retainer to maintain the extraction phase, a method known as hollow fiber liquid phase micro extraction (HF–LPME).[12,13]

HF–LPME can be performed in two or three micro-extraction phase modes. The three phases involved in the extraction are the analyte solution (donor), an organic phase and second aqueous phase into which the extraction is conducted (acceptor phase). During the extraction, the desired analyte under proper conditions is first transferred into the organic phase and then into the acceptor phase. The rate of conducting the extraction depends on the rate of mass transfer between the two interfaces of the donor/organic phase and the organic/acceptor phase. The main reported limitation of this method is that it is only applicable for species which are capable of ionization.[14,15] In the present study, a simple, inexpensive and sensitive three-phase hollow fiber micro-extraction technique combined with high performance liquid chromatography-ultraviolet detection (HPLC–UV) is described for the extraction and determination of STG in urine samples.

EXPERIMENTAL

Chemicals and materials

Sitagliptin reference standard was kindly donated by the Food and Drug Organization (Tehran, Iran). A stock solution containing 200 μg mL^{-1} of sitagliptin was prepared in HPLC grade methanol and stored at 4 °C. Working solutions (from 2 to 300 μg L^{-1}) were freshly prepared in HPLC grade water by dilution of the stock solution and filtered using a Millipore

filter (0.45 μm) each day prior to use. All chemicals were of analytical reagent grade unless otherwise stated. The urine samples were obtained from the Taleghani Clinic (Tehran, Iran). The samples were diluted using deionized water and were used for method development and calibration. The working standards for real sample analysis were prepared by spiking STG to 15 mL of urine samples, which were diluted 1:1 with ultra-pure water.

Apparatus and chromatographic conditions

The chromatographic separations were realized on a Youngling HPLC instrument equipped with a YL 9110 quaternary HPLC pump, a 20 μL sample loop and YL 9120 UV-Vis detector. The chromatographic data were collected and analyzed using a Younglin Autochro-3000. The separations were performed on a C_{18} column (150 mm×4.6 mm, with 5 μm particle size) from Teknokroma (Barcelona, Spain). A mixture of methanol and 0.1 % perchloric acid (40:60) at a flow rate of 1.0 mL min^{-1} was used as the mobile phase. The injection volume was 20 μL for all the standards and samples, and the detection was performed at a wavelength of 268 nm. The pH measurements were performed using a GPHR 1400A pH meter (Berlin, Germany). A magnetic stirrer model MR Hei-Standard (Heidolph Company, Germany) and a 25 μL syringe model 702 NR from Hamilton (Bonaduz, Switzerland) were also used. The ultrapure water was from a MilliQ Ultrapure water purification system (Millipore, Bedford, MA, USA). All extractions were realized using a Q3/2 Accrual polypropylene hollow fiber membrane from Membrana (Wuppertal, Germany) with a 0.2 μm pore size, 600 μm internal diameter and 200 μm wall thickness.

Extraction procedure

Initially, the polypropylene fibers were cut to the size of 8.5 cm (the volume of these fibers was approximately 22 μL). The fibers were completely washed with acetone in an ultrasonic bath for 10 min and then dried at room temperature before use for extraction. In order to avoid any possible memory effects, a new fiber was used for each extraction. In the extraction procedure, 15 mL of the sample solution with a known concentration and alkaline pH was transferred to a 20-mL sample vial containing a magnetic stirring bar. The sample vial was placed on a Heidolph MR 3001 K magnetic stirrer. Thirty μL of an acceptor phase with an acidic *pH* was withdrawn by a 25 μL Hamilton micro-syringe and the syringe needle was inserted into the hollow fiber segment. The fiber was immersed in an organic solvent for 10 s (*n*-octanol) to impregnate the fiber pores with the organic solvent. The fiber was then placed in distilled water for 30 s in order to wash the extra solvent from its surface. The acceptor phase inside the syringe was injected into the lumen of the fiber. The end of the fiber was blocked by a piece of aluminum foil. The U-shaped fiber was placed in the sample vial. The sample solution was agitated at 1000 rpm. At the end of the extraction (after 50 min), the fiber was removed from the sample solution and the acceptor phase was then retracted back into the syringe. Finally, the extraction phase (20 μL) was injected directly into the HPLC system in order to analyze the drug. In 2006, Pawliszyn and co-workers successfully automated the HF–LPME technique with a CTC CombiPal autosampler, and a new device was designed for the automation of HF–LPME in this study.[16]

RESULTS AND DISCUSSION

In this study, a three-phase hollow-fiber micro-extraction technique combined with HPLC–UV was used for the extraction and determination of STG in biological samples. Furthermore, the effect of different experimental parameters on the efficiency of extraction, such as the type of organic solvent, the pH of the

donor and acceptor phases, the stirring time, the ionic strength of the donor phase, temperature, the stirring rate and the volume ratio of donor phase to acceptor phase were all investigated and optimized to obtain the best analytical performance.

Type of organic solvent

It is important to select a suitable solvent in order to achieve maximum extraction, good sensitivity, selectivity and high accuracy. The chosen solvent should enter easily into the fiber pores, be non-toxic, and ultimately have a high boiling point (be non-volatile) and a proper viscosity to be stable during the extraction process.[10,17,18]

In order to select a proper solvent, several solvents (*n*-octanol, *n*-octane, 1-decanol, 4-methyl-2-pentanone and benzyl alcohol) were examined. The highest extraction efficiency was obtained using *n*-octanol (Fig. 1).

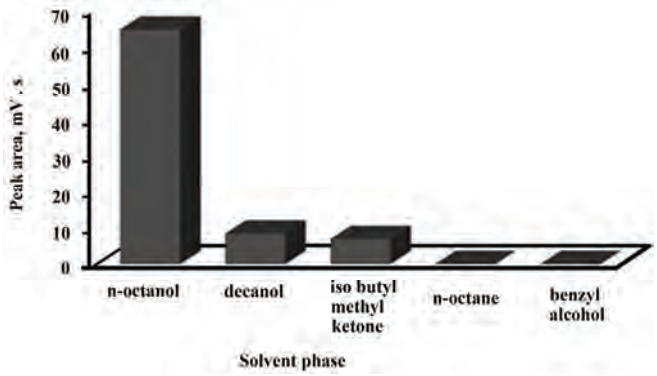

Fig. 1. The effect of organic solvent on HF–LPME efficiency in the determination of sitagliptin.

pH of acceptor phase and donor phase

The pH values of the donor and acceptor solutions are important factors in a three-phase LPME. For ionizable analytes, acid–base dissociation is the most common reaction utilized to facilitate the extraction of the analyte from the donor to the acceptor phase.[18–20] The pH difference between the donor and acceptor phases can promote the transfer of analytes. For practical applications, the pH should differ from the pK_a value of the analyte (the pK_a of STG is 7.7) by at least 3 units.[21]

To promote extraction, the pH of the donor solution was adjusted in order to deionize the analyte. Therefore, the effect of the donor phase pH on the extraction efficiency was studied by changing the sample pH from 8.0 to 12.5. The maximum efficiency of the analyte extraction was achieved at pH 10.5.

The effect of the pH of the acceptor phase from 2.0 to 4.0 was studied. At pH of 3, the extraction rate increased because STG had an ionic structure at this pH and transferred easily into the aqueous phase.

Accordingly, pH values of 10.5 and 3 were selected as optimum values for the donor and acceptor phases respectively.

Stirring rate of analyte solution

Stirring the sample during extraction reduces the thermodynamic equilibrium time and increases the extraction efficiency as stirring provides a fresh donor solution for the organic phase to extract and enhances analyte transport from the donor phase to the organic solvent. To evaluate the effect of sample stirring, the aqueous donor sample was stirred for 50 min at different rates (from 250 to 1200 rpm). The efficiency of extraction increased with increasing stirring speed. However, at 1200 rpm there was a decrease in the extraction efficiency, probably due to the high speed creating air bubbles on the surface of the hollow fiber that ultimately leads to poorer precision and possible failure of the experiment.[10,22,23] Finally, a stirring speed of 1000 rpm was adopted for the subsequent experiments.

Ionic strength of the donor phase

In this stage, the effect of adding salt to the donor phase on the extraction rate was investigated. Generally, two phenomena occur simultaneously when salt is added to water. The first phenomenon is known as salting-out (this can increase the efficiency of the extraction) and the second phenomenon is electrostatic interactions between the analyte molecules and the salt,[18,19] which reduces the mobility of the analyte molecules and increases the thickness of the emission layer between the sample solution and fibrated organic layer, that reduces the extraction efficiency. Furthermore, the addition of salt could increase the viscosity of the sample solution and change the physical properties of the fiber wall, which could reduce the movement of analyte from the donor to solvent phase. Overall, it is fair to comment that the addition of salt depends on the nature of the analyte and could increase, decrease or, in some cases, not change the efficiency of extractions.[24,25]

In this study, the effect of salt addition (NaCl) from 0 to 350 g L^{-1} was investigated on the extraction rate. NaCl addition significantly increased the drug extraction. The concentration of 350 g L^{-1} was selected as the optimum value for further studies.

Thus, according to the nature, structure and the pK_a value (7.7) of STG, the salting out phenomenon was dominant in this process and, therefore, increasing the salt concentration, increased the extraction rate of the drug.

Extraction time

The extraction and recovery of an analyte is affected by the stirring time of the sample solution, which facilitates the transfer of an analyte from the donor phase to the organic phase and finally to the acceptor phase. In this study, the effect of extraction time between 20 and 60 min was examined.

Normally, if the extraction time is too long, the loss of solvent and the formation of air bubbles may occur, both of which affect the extraction efficiency.[17,10,26,27]

The obtained results showed that with increasing time, the extraction rate increased. Based on thee results, an extraction time of 50 min was selected as a suitable extraction time.

Temperature

In this study, the effect of temperature on extraction rate was investigated in the range from 22 to 45 °C. The results demonstrated that the extraction efficiency was reduced on increasing the temperature. This could be due to solvent evaporation and bubble formation on the fiber wall.[19] Therefore, 25 °C was chosen as the optimum temperature for the extractions.

Volume ratio of donor phase to acceptor phase

In the three phase HF–LPME, an analyte transfers from an aqueous donor phase to an organic solvent and finally to an acceptor phase existing in the fiber during an emission process. Pre-concentration factor (*PF*) in this process is calculated based on the equation:

$$PF = \frac{100 V_{\mathrm{d}} R}{V_{\mathrm{a}}}$$

where V_{d} and V_{a} are the volumes of the donor phase and acceptor phase, respectively, and R is the extraction efficiency.[20,25]

In this study, the volume of the acceptor phase was kept constant (20 μL), and the volume of donor phase was altered and the effects of these changes on the extraction rate were investigated. Based on the obtained results, 15 mL was selected as the optimum volume of the donor phase.

Validation method

The calibration curve for STG in aqueous solutions was plotted in the concentration range from 0.05 to 10 mg L^{-1}. For each level, three replicate extractions were performed under the optimum pre-concentration conditions (Table I).

TABLE I. Optimum conditions for the pre-concentration of sitagliptin

*V*d / mL	*T* / °C	[NaCl] / g dL^{-1}	*t* / min	Stirring rate, rpm	pH of AP	pH of DP	Solvent
15	25	35	50	1000	3	10.5	*n*-Octanol

The corresponding regression equation, coefficient of determination (R^2), dynamic linear range (*DLR*), limit of detection (*LOD*) and pre-concentration factor (*PF*) of STG were calculated and are summarized in Table II. The *LOD* was calculated at a signal-to-noise ratio of 3 and was obtained by adding the standard STG solution to drug-free urine samples. The results indicated that the proposed method displayed good reproducibility to determine sitagliptin with intra-day *RSD* value of 3.4 % and inter-day *RSD* value of 5.25 % (for 3 consecutive days, the extraction was performed at an STG concentration of 1 mg L^{-1}). Under the optimized conditions, enrichment factors of up to 88 were achieved.

TABLE II. The values of the corresponding regression equation and the analytical characteristics of the proposed HF–LPME method; *PF*, pre-concentration factor; *LOD*, limit of detection; *LOQ*, limit of quantification; R^2, coefficient of determination; *DLR*, dynamic linear range and *RSD* %: relative standard deviation; Analyzed concentrations, mg L^{-1}: 0.05, 0.2, 0.5, 3, 5, 7, 9 and 10

Inter-day *RSD* / %	Intra-day *RSD* / %	*DLR* range ng mL^{-1}	R^2	Intercept	Slope mV s L mg^{-1}	*LOQ* ng mL^{-1}	*LOD* ng mL^{-1}	*PF*
5.25	3.4	2.5–200	0.999	25.881	328.09	3.0	1.0	88

Extraction of sitagliptin from urine samples

Under the optimized conditions, the developed HF–LPME–HPLC technique was applied to the pre-concentration and determination of sitagliptin in urine samples. The pH of the real samples was adjusted to 10.5 by the addition of 0.1 mol L^{-1} NaOH solution. Prior to the spiking and extraction of the target drug, the urine samples were diluted 1:1 with water. Typical chromatograms for spiked and non-spiked urine samples obtained by the HF–LPME–HPLC method are shown in Figs. 2 and 3, respectively.

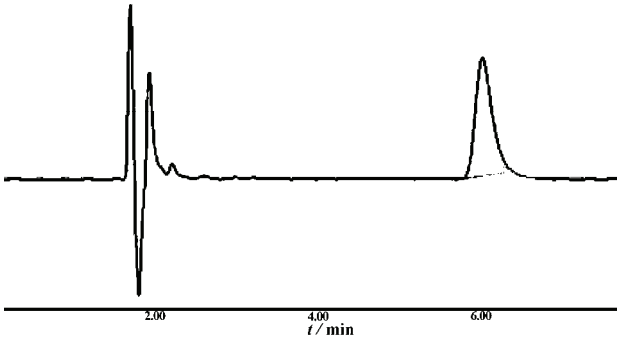

Fig. 2. Representative HF–LPME–HPLC chromatograms of urine sample spiked with 10 ng/mL of sitagliptin under the optimum conditions (*n*-octanol as organic solvent; the donor phase *pH* of 10.5; the acceptor phase *pH* of 3; stirring speed of 1000 rpm; concentration of NaCl 35 g dL^{-1}; extraction time of 50 min; temperature of 25 °C; volume of donor phase of 15 mL).

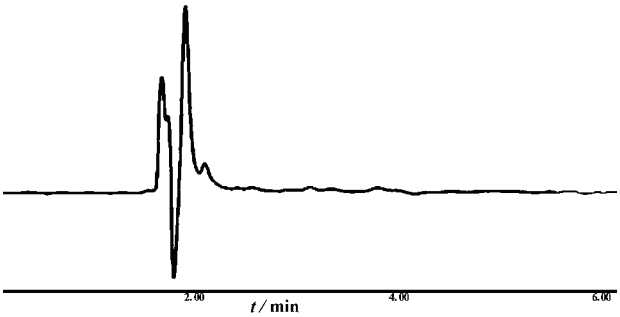

Fig. 3. Representative HF–LPME–HPLC chromatograms of control urine sample.

Comparison of the present method with other reported methods

The present method was compared with other studies in terms of the method of extraction, validation, and precision. The method compares well with those mentioned in Table III. As can be seen, the *LOD* value of this method is comparable with those obtained in previous studies, and even lower than those reported in the literature. In addition, due to the simplicity and low cost of the extraction device, the hollow fiber, it can be discarded after each extraction to avoid carry-over and cross-contamination.

TABLE III. Comparison of the proposed method with other developed methods for determination of sitagliptin in biological samples

Determination method	Extraction method	*LOD* μg mL^{-1}	*DLR* μg mL^{-1}	*LOQ* μg mL^{-1}	*RSD* %	Real sample	Ref.
HPLC–UV	HF–LPME	0.001	0.0025–0.2	0.003	3.4	Urine	Present work
HPLC	LLE	–	0.75–100	0.75	10>	Plasma	28
LC/MS/MS	LLE	–	0.001–1	0.001	2–6.1	Plasma	6
HTLC/MS/MS	TFO[a]	–	0.1–50	0.1	2.3–6.5	Urine	29
RP-LC	–	0.02	0.25–200	0.075	0.93–1.62	Plasma	30
LC/MS/MS	LLE	–	0.001–0.25	0.001	<6	Plasma	7
LC	MISPE[b]	0.03 (μg L^{-1})	0.1–100 (μg L^{-1})	0.1 (μg L^{-1})	0.4	Urine	8

[a]TFO: turbulent flow online, [b]MISPE: molecularly imprinted solid-phase extraction

CONCLUSIONS

This work introduced a three-phase HF–LPME method combined with HPLC–UV for the extraction, pre-concentration and determination of STG in urine samples. The LPME technique proved to have several advantages over the other extraction methods such as liquid–liquid extraction (LLE) and solid phase extraction (SPE). A simple, fast and inexpensive HF–LPME–HPLC–UV with

high accuracy and good sensitivity for the extraction and determination of STG in human urine samples was developed and validated.

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ИЗВОД

ЕКСТРАКЦИЈА ШУПЉИМ ВЛАКНИМА КОМБИНОВАНА СА HPLC ЗА ОДРЕЂИВАЊЕ СИТАГЛИПТИНА У УЗОРЦИМА УРИНА

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У овом раду је успешно развијена трофазна микроекстракција шупљим влакнима у течној фази (HF–LPME) спрегнута са високоефикасном течном хроматографијом (HPLC) за одређивање трагова лека, антидијабетика ситаглиптина (STG), у узорцима урина. Ситаглиптин је екстрахован из 15 mL основног раствора, на pH 8,5 у органски растварач (*n*-октанол) у порама шупљег влакна и ре-екстархован у закишељен раствор у лумену шупљег влакна на pH 3. После екстракције, 20 μL акцепторске фазе је инјектирано у HPLC систем. У циљу боље ефикасности екстракције, испитивани су и оптимизовани параметри који утичу на HF–LPME, као што су pH донорске и акцепторске фазе, тип органске фазе, јонска јачина, брзина мешања, време екстракције, запремински однос донорске и акцепторске фазе и температура. Под оптималним условима је постигнут фактор преконцентрисања од 88 и добијена релативна стандардна девијација у опсегу 3–6 %. Резултати указују да HF–LPME метода има одличан капацитет за пречишћавање и висок фактор преконцентрисања, па може бити примењена као једноставна и осетљива метода за праћење лекова у узорцима урина.

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REFERENCES

1. R. Pathak, M. B. Bridgeman, *P&T* **35** (2010) 509
2. D. K. Badyal, J. Kaur, *JK Science* **10** (2008) 97
3. E. Fleury-Milfort, *J. Am. Acad. Nurse Pract.* **20** (2008) 295
4. A. J. Scheen, *Diabetes Obes. Metab.* **12** (2010) 648
5. S. Terabe, *Anal. Chem.* **76** (2004) 241A
6. W. Zeng, Y. Xu, M. Constanzer, E. J. Woolf, *J. Chromatogr., B* **878** (2010) 1817
7. R. Nirogi, V. Kandikere, K. Mudigonda, P. Komarneni, R. Aleti, R. Boggavarapu, *Biomed. Chromatogr.* **22** (2008) 214
8. R. Rao, P. Maurya, S. Khalid, *Talanta* **85** (2011) 950
9. A. Rodríguez, S. Pedersen-Bjergaard, K. E. Rasmussen, C. Nerín, *J. Chromatogr., A* **38** (2008) 1198
10. K. M. Al Azzama, A. Makahleaha, B. Saada, S. Mansorb, *J. Chromatogr., A* **1217** (2010) 3654
11. Y. Tao, J. F. Liu, X. L. Hu, H. C. Li, T. Wang, G. B. Jiang, *J. C hromatogr., A* **1216** (2009) 6259
12. S. Pedersen-Bjergaard, K. E. Rasmussen, *Anal. Chem.* **71** (1999) 2650

13. K. E. Rasmussen, S. Pedersen-Bjergaard, M. Krogh, H. G. Ugland, T. Grønhaug, *J. Chromatogr., A* **873** (2000) 3
14. K. E. Rasmussen, S. Pedersen-Bjergaard, *Anal. Chem.* **23** (2004) 1
15. L. Hou, H. K. Lee, *Anal. Chem.* **75** (2003) 2784
16. G. Ouyang, J. Pawliszyn, *Anal. Chem.* **78** (2006) 5783
17. S. Shariati, Y. Yamini, A. Esrafili, *J. Chromatogr., B* **877** (2009) 393
18. E. Tahmasebi, Y. Yamini, A. Saleh, *J. Chromatogr., B* **877** (2009) 1923
19. M. Saraji, M. T. Jafari, H. Sherafatmand, *J. Chromatogr., A* **1217** (2010) 5173
20. H. Ebrahimzadeh, Y. Yamini, H. A. Firozjaei, F. Kamarei, N. Tavassoli, M. R. Rouini, *Anal. Chim. Acta* **665** (2010) 221
21. S. Pedersen-Bjergaard, K. E. Rasmussen, *J. Chromatogr., A* **1184** (2008) 32
22. J. Xiong, J. Chen, M. He, B. Hu, *Talanta* **82** (2010) 969
23. M. Saraji, B. Farajmand, A. A. Ensafi, A. R. Allafchian, Z. M. Zare, *Talanta* **82** (2010) 1588
24. Y.-J. Dong, *J. Chin. Chem. Soc.* **56** (2009) 549
25. M. Liu, B. Qiu, X. Jin, L. Zhang, X. Chen, G. Chen, *J. Sep. Sci.* **31** (2008) 622
26. F. Barahonaa, A. Gjelstad, S. Pedersen-Bjergaard, K. E. Rasmussen, *J. Chromatogr., A* **1217** (2010) 1989
27. Y. Yang, J. Chen, Y. P. Shi, *J. Chromatogr., B* **878** (2010) 2811
28. X. F. Jiu, D. W. Shang, Y. Chen, X. G. Li, X. M. Wan, T. Y. Zhou, W. Lu, *J. Chin. Pharm. Sci.* **20** (2010) 63
29. W. Zeng, D. Musson, A. Fisher, L. Chen, M.S. Schwartz, E. J. Woolf, *J. Pharm. Biomed. Anal.* **46** (2008) 534
30. R. El-Bagary, E. F. Elkady, B. M. Ayoub, *Talanta* **85** (2011) 673.