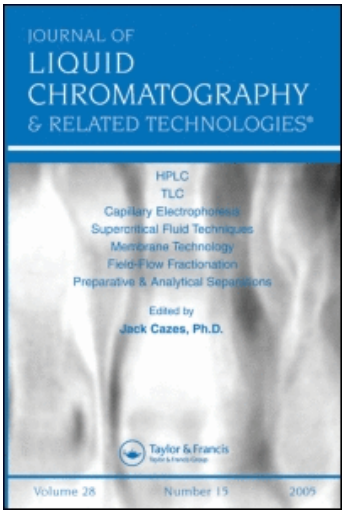

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

SIMPLE GENERIC SPE ASSAY FOR HPLC ANALYSIS OF MORPHINE AND ITS GLUCURONIDES IN SERUM SAMPLES

Eva Brandšteterová[a]; Eva Blahová[a]; Jana Netriová[b]
[a] Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Bratislava, Slovakia
[b] National Institute of Oncology, Bratislava, Slovakia

Online publication date: 09 March 2002

To cite this Article Brandšteterová, Eva , Blahová, Eva and Netriová, Jana(2002) 'SIMPLE GENERIC SPE ASSAY FOR HPLC ANALYSIS OF MORPHINE AND ITS GLUCURONIDES IN SERUM SAMPLES', Journal of Liquid Chromatography & Related Technologies, 25: 16, 2521 — 2534
To link to this Article: DOI: 10.1081/JLC-120014272
URL: http://dx.doi.org/10.1081/JLC-120014272


MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
Vol. 25, No. 16, pp. 2521–2534, 2002

SIMPLE GENERIC SPE ASSAY FOR HPLC ANALYSIS OF MORPHINE AND ITS GLUCURONIDES IN SERUM SAMPLES

Eva Brandšteterová,[1,*] Eva Blahová,[1] and Jana Netriová[2]

[1]Department of Analytical Chemistry,
Faculty of Chemical and Food Technology,
Radlinskeho 9, 812 37 Bratislava, Slovakia
[2]National Institute of Oncology, Klenova 1,
833 10 Bratislava, Slovakia

ABSTRACT

A very simple solid-phase extraction (SPE) generic assay has been developed for high performance liquid chromatography (HPLC)-DAD or HPLC-UV analysis of morphine and its two metabolites (morphine-3-glucuronide and morphine-6-glucuronide) in serum samples of cancer patients. Solid-phase extraction cartridges with OASIS polymeric hydrophilic–lipophilic sorbent have been applied for SPE off-line procedure. High extraction recoveries have been obtained for simultaneous isolation of all three analytes: 90% for morphine, and 96% and 99% for morphine-3-glucuronide M3G and morphine-6-glucuronide M6G, respectively.

The main goal of this work was to optimize loading, washing, and elution steps of the SPE assay, especially pH of loaded

*Corresponding author. E-mail: branstet@cvt.stuba.sk

DOI: 10.1081/JLC-120014272
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

sample, volumes of applied wash, and elution solvents with the aim to reduce time for sample preparation and to simplify the SPE procedure. Sorbents of a new generation has also been applied in the HPLC assay (Symmetry Shield RP-8) with mobile phases without any ion-pair agent, and without or with a very low content of organic modifier. The UV detection at 210 nm was used and the following detection limits were achieved: morphine 6.9 ng/mL, M3G 2.3 ng/mL, and M6G 20.0 ng/mL. The developed method has been recommended for routine monitoring of morphine and its glucuronides, in serum samples of cancer patients in clinical laboratories.

INTRODUCTION

Morphine (M) is recommended by the World Health Organization as the opioid of choice for moderate to severe cancer pain. The major pathway of morphine clearance is via glucuronidation in the liver, predominantly to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Morphine-6-glucuronide appears to be more potent than morphine as an analgesic agent, and M3G does not have analgesic activity.[1] The metabolic pathway of morphine is illustrated in Fig. 1.[2] Monitoring of morphine and its two glucuronides is

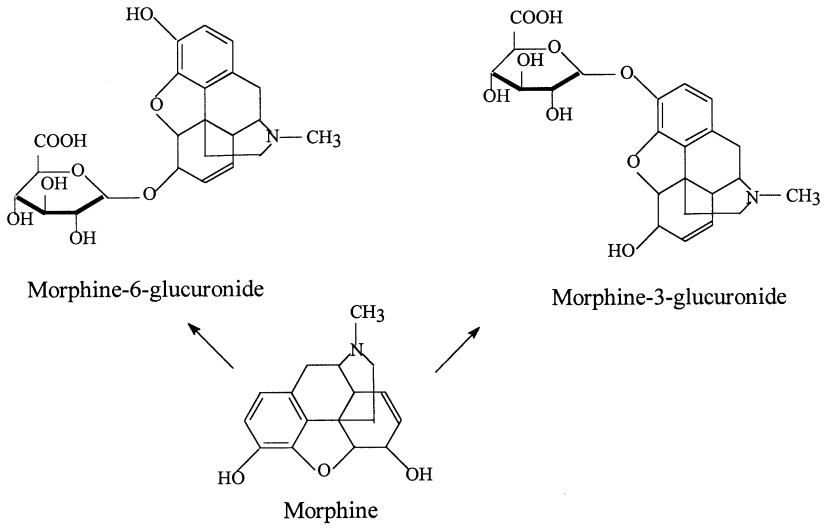

Figure 1. Metabolic pathways of morphine.

important, not only for the study of the correlation between applied doses of drug and analgesic effect, but also for the study of metabolic rate. The ratio of morphine and its glucuronide concentrations or ratio of both metabolites enable the characterization of patients with "fast" or "slow" metabolism.

High performance liquid chromatography (HPLC), in combination with various detection modes, is the method of choice for the simultaneous analysis of morphine and its metabolites. Immunoassays or radio-immunoassays are very sensitive techniques, but cross-reactions of all analytes with the anti-sera could cause false positive or negative results.

Developed HPLC assays have used UV, DAD, fluorescence, and electrochemical detectors or their combinations. Hyphenated LC-MS or LC-MS-MS techniques could simplify metabolite identification a great deal, but these very expensive instruments are not available commonly in hospital laboratories. So, the HPLC assay, which could provide a simple and fast analysis, is very often appreciated for routine clinical monitoring.

The preparation step of serum samples is very important part of the HPLC assay, as it is very time consuming and laborious, and it could influence results of the complete analysis. Therefore, it is very important to develop simple, fast, and effective methods for serum sample preparation, with high recoveries for all analyzed compounds. Solid-phase extraction (SPE) provides good possibilities for sample clean-up and preconcentration of analytes. Solid-phase preparation has often been recommended for the extraction of morphine and its metabolites from serum. However, both morphine glucuronides are highly polar in comparison to morphine. Moreover, the great differences in pK values of all three analytes very often cause difficulties in the SPE method development. The hyphenation of HPLC with MS could simplify the SPE procedure significantly, as demands on the extract purity are not so strict as for the commonly applied HPLC assays. More interfering or coeluting compounds cause less problems in HPLC-MS technique, because the chromatographic resolutions of all analytes does not have be optimal for their detection and identification. But, in some papers, relatively complicated SPE assays also have been described for HPLC-MS of morphine and its glucuronides.[3–7] Automatic SPE in off-line mode and 96-well SPE have also been tested.[8,9]

For the HPLC method with commonly applied detectors, more attention has to be devoted to the SPE procedure. Different sorbents have been tested for SPE procedures and the most of published methods have used very complicated and time consuming sample handling steps.[10–13] Commonly available reversed-phase cartridges have been used for the optimization of the SPE steps, including time for drying sorbent before both washing and elution steps.[14] Automatic on-line SPE has already been described for morphine and its glucuronides determination in plasma and cerebrospinal fluid using the sample preparator OSD-2.[15]

The development of an immunoaffinity-based sample pretreatment for the determination of morphine and its glucuronides in human blood has also been

described.[16] The resulting extracts were analyzed by HPLC with native fluorescence detection. The mean recoveries from spiked blood samples were 71%, 76%, and 88% for morphine, M3G and M6G, respectively.

The aim of this work, was to develop the simple generic SPE assay before HPLC analysis of M, M3G, M6G in serum samples using new polymeric sorbent (OASIS HLB). Special attention has been given to the optimization of washing and elution steps for serum preparation. Symmetry Shield RP-8 column has been chosen for HPLC analyses. The developed method has been applied in routine monitoring of morphine and its glucuronides in serum samples of cancer patients.

EXPERIMENTAL

Chemicals and Solutions

Acetonitrile, methanol (HPLC grade), were supplied by Merck (Slovakia). KCl (p.a.), NaCl (p.a.), H_3PO_4 (p.a.), $Na_2HPO_4 \cdot 2H_2O$ (p.a.), $NaH_2PO_4 \cdot H_2O$ (p.a.), were from Lachema Brno (Czech Republic). Standards of morphine hydrochloride was obtained from National Institute of Oncology (Slovakia), M3G from KEÚ MV (Slovakia), M6G from Lipomed (Switzerland).

Isotonic solution (PBS—Phosphate-Buffered Saline) was prepared dissolving 0.2 g of KCl, 8.0 g of NaCl, 0.2 g of KH_2PO_4 and 1.15 g of Na_2HPO_4 in 1 L of deionized water. pH value was adjusted to 7 with H_3PO_4.[17] Standard stock solution of morphine (101 mg/mL) was prepared in methanol, stock solutions of M3G (60 mg/mL) and M6G (100 mg/mL) in the mixture of methanol and water (1 : 1). Working solutions were prepared by diluting the stock solutions with deionized water. The stability of the working solutions was checked every week. All solutions were stored in the refrigerator at 5°C and were stable at all times.

Equipment

A sonicator Sonorex RK-100H, Bandelin electronic (Germany), and a laboratory centrifuge MPW-300 Mechanika precyzyjna (Poland), were used for sample preparation and HPLC analysis. OASIS HLB cartridges (30 mg, 1 mL), Waters (USA) were applied for the SPE procedure of standard mixtures and serum samples.

The HPLC system consisted of an HPLC pump Delta Chrom SDS 030, Watrex (Slovakia), an injector Rheodyne 7125 (20 mL loop), (USA), an autosampler Basic-Marathon, Spark Holland, Watrex (Slovakia), an UV VIS detector 484, Waters Corp. (USA), and a DAD detector HP 1100, Hewlett Packard (USA). Data were collected using CSW 1.7 software Microsoft (USA).

For HPLC separations, a Symmetry Shield RP8 (150 × 4.6 mm, 5 mm) reversed-phase column, Waters (USA) was used.

Chromatographic Conditions

The mobile phases consisting of 20 mM phosphate buffer (adjusted to pH 2.5; 6.5 with phosphoric acid) with acetonitrile (0; 1%) ($F = 1.0$ mL/min) were tested for HPLC separation of morphine and its metabolites.

Detection was realized using a UV detector at 210 nm and DAD detector.

Sample Preparation

The blank serum and the clinical serum samples were obtained from National Institute of Oncology (Slovakia).

To 1.5 mL of blank serum, stock solutions of morphine, M3G and M6G were added to prepare samples with concentrations of 2.0; 0.9 and 1.0 mg/mL, respectively, and the samples were homogenized using the sonicator.

Spiked blank serum and clinical serum samples were centrifuged for 15 min at 3000 rpm. For the SPE procedure, the volume of 0.5 mL of serum was used. Solid-phase extraction procedures for standard mixtures and serum samples were the following:

Solid-phase extraction for standard mixture:

I. 1 mL methanol
   1 mL water
   0.5 mL water standard mixture
   0.5 mL water
   0.5 mL methanol

II. 1 mL methanol
   1 mL water
   0.5 mL standard mixture in PBS
   0.5 mL water
   1 mL methanol

Solid-phase extraction for serum samples:

III. 1 mL methanol
   1 mL water
   0.5 mL serum sample
   0.5 mL, 1 mL, 3 mL, 5 mL water
   1 mL methanol

The eluates were evaporated to dryness and residues were reconstituted in 0.5 mL of mobile phase.

RESULTS AND DISCUSSION

Two reversed-phase columns, with sorbents of new generation (Symmetry Shield RP-8 and XTerra C-18), were chosen for HPLC assay in our previous work, after evaluating chromatographic characteristics (retention times, capacity factors, chromatographic resolutions, asymmetry factors, and numbers of theoretical plates) for M, M3G, and M6G.[18] Recommended mobile phases could be applied in the wide pH range (2.5–9.5), without or with very low content of organic modifier (0, 1, 2% of acetonitrile), and without any ion-pair agents. The same HPLC conditions have also been used in this paper for the simple generic SPE method development and the analysis of morphine and its glucuronides in serum samples. All HPLC analyses were realized with the UV detector and quantitative analysis was performed at 210 nm. Detection limits at signal-noise ratio 3 were determined at this wavelength as follows: M: 6.9 ng/mL, M3G: 2.3 ng/mL and M6G: 20.0 ng/mL. Linear responses of UV detector were evaluated in concentration ranges for M. 0.01–2.0 mg/mL, M3G: 0.1–20.0 mg/mL, M6G: 0.1–5.0 mg/mL.

The main part of this work was devoted to the optimization of the SPE serum preparation before the HPLC analysis. According to the cited literature references, most of the authors have described relatively complicated and time consuming SPE assays with the applications of commonly available reversed-phase cartridges.[3,10–12] In some cases, two step SPE procedures have also been developed[13] with the combination of reversed-phase and ion-exchange sorbents.

The great problem in the SPE method development is caused by the different polarities and pK values of all three analyzed compounds. Both glucuronides are very polar, their retentions on the reversed-phase SPE sorbent are very poor and pH values for all SPE steps (cartridge conditioning, sample loading, interference washing, and analyte elution) have to be carefully optimized. The SPE method is significantly more complex because analytes with so different properties have to be isolated simultaneously. Morphine has a ternary amine with pKa of 7.9 and a phenolic hydroxyl group at the 3′carbon with pKa of 9–10. Both glucuronides have a carboxylic group with pKa in the range 3–4, but M3G has lost the phenolic hydroxyl group.[7] For these reasons, the developed SPE method has to employ a fine balance between pH and polarity manipulation to achieve retention and elution of all analytes.

Recently, a new material was developed for SPE clean-up procedure. This hydrophilic–lipophilic balanced (HLB) macro-porous polymer sorbent enables obtaining good recoveries (greater than 85%) and excellent reproducibility values (less than 5.5% R.S.D.) for wide range of compounds, including acids, neutrals, bases, parent compounds, and polar metabolites.[19] Additionally, this HLB sorbent is fully wettable with water, therefore, it is not necessary to include any sorbent drying steps.

In this work, OASIS HLB cartridges have been applied for M, M3G, and M6G simultaneous isolation from serum samples. At first, a simple generic assay recommended by the manufacturer,[17] has been tested for the standard mixture. In this generic method, only the concentration of the organic solvent was manipulated in the SPE procedure: 1 mL methanol, 1 mL water, 0.5 mL sample, 0.5 mL water, 0.5 mL methanol, evaporation to dryness, dissolution in the mobile phase. The contents of all three analytes were evaluated in all SPE steps and results are demonstrated in Table 1. As is obvious, the simple generic SPE assay could be sufficient for the simultaneous isolation of M and M6G. But, extraction recovery of morphine is relatively low (70%), which is caused by using a small volume (0.5 mL) of methanol in the elution step. It is supposed, that morphine is strongly retained at HLB sorbent and the applied volume of methanol is not sufficient for its elution. Moreover, it was found out, that 6–10% of M3G were lost after loading the sample onto the cartridge. About 40% of M3G were washed from the sorbent in the washing step and about 45% of M3G were eluted in the elution step. This means, that only 45% extraction recovery for M3G was achieved using a simple generic SPE assay. These results have confirmed the fact, that simple generic SPE assay, in some cases, has to be slightly modified for the simultaneous isolation of analytes with very different chemical properties.[19]

So, in the second SPE assay, the influence of pH in the sample loading step has also been taken into consideration. The standard mixture was loaded onto the SPE cartridge at pH similar to pH of serum samples. Standards were dissolved in PBS solution (pH = 7) and the volume of elution solvent (methanol) was higher (1 mL). As is possible to see in Table 2, extraction recoveries for this simply modified SPE scheme are very high for all analytes.

As serum samples have similar pH value, no pH regulation has been done for the serum sample loading. So, in this case, serum sample has been applied directly onto the SPE cartridge. But, after the application of serum on the HLB SPE cartridge, it was found out, that the volume of 0.5 mL water is not sufficient for removing all interfering components from serum and some of them could coelute with analyzed compounds. High performance liquid chromatograms of

Table 1. Content of Analytes in Individual Steps of SPE Procedure I ($n = 4$)

Content of Analytes (%) Fraction	M3G	RSD (%)	M6G	RSD (%)	M	RSD (%)
0.5 mL of water standard mixture	8.4	4.7	—	—	—	—
0.5 mL of water	40.5	5.0	—	—	—	—
0.5 mL methanol	44.5	1.7	94.5	3.7	68.6	1.8

Table 2. Extraction Recoveries of Analytes for SPE Procedure II ($n = 4$)

Analyte	Extraction Recovery (%)	RSD (%)
M3G	98.6	1.2
M6G	98.7	1.2
M	94.5	0.7

standard mixture (a) and blank serum (b), are illustrated in Fig. 2. It is obvious, that peaks eluting before morphine could complicate its quantification. Therefore, higher volumes of water (1, 2, 3 mL) have also been tested in the washing step with the aim to minimize this interfering peak from serum. For the HPLC analysis of SPE extracts, Symmetry Shield RP-8 column and mobile phase: 0% or 1% of acetonitrile in phosphate buffer (pH = 6.5) were used. Morphine recovery was 79.5% for the washing volume of 1 mL water. Increasing the washing volume (2.3 mL), has increased morphine recovery, but recoveries of both glucuronides

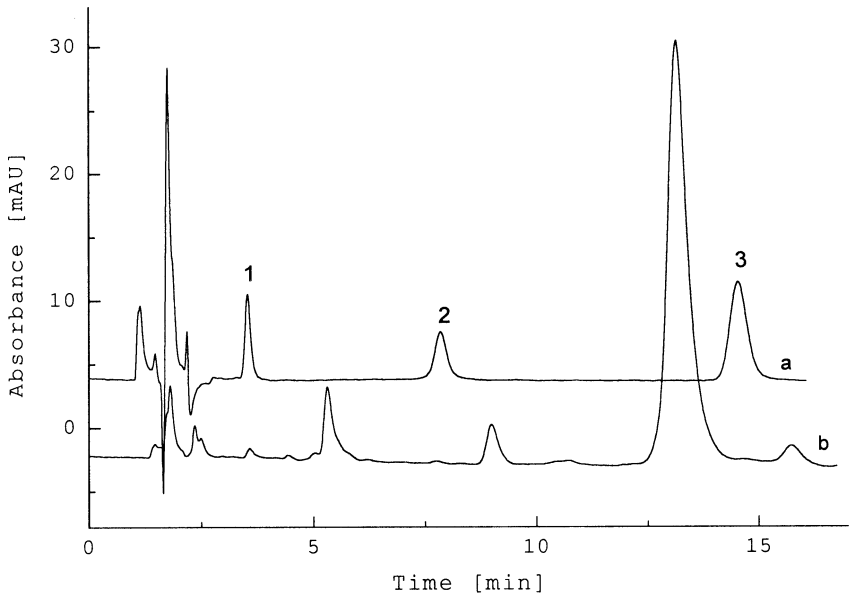

Figure 2. High performance liquid chromatogram of standard mixture of M3G, M6G, M (a) and blank serum (b) High performance liquid column: Symmetry Shield RP8, mobile phase: 1% acetonitrile + 99% 20 mM Na_2HPO_4, pH 6.5; $F = 1.0$ mL/min. Detection: UV, 210 nm, injection volume: 20 μL. Separation order: 1-M3G, 2-M6G, 3-M.

have decreased significantly (Table 3). Therefore, it is necessary to make a decision as to what volume of water should be chosen for a washing step in the SPE assay of clinical samples. In the case of analytes being present in higher concentration (as it is common for cancer patient samples), it is possible to also choose the assay with lower recoveries but better removal of interfering compounds from biological material. Using 2 mL or 3 mL of water in the washing step, the peak eluting before morphine is decreasing and chromatographic resolution of morphine and this compound is better, as it is possible to see from Fig. 3.

Better separation of interfering peaks and morphine could also be achieved using the mobile phase without any content of acetonitrile.[18] In this case, chromatographic resolution has increased and it is sufficient for the quantitative analysis. Therefore, for SPE procedures of clinical samples, volumes of 1 mL or 2 mL of water were chosen for a washing step; and mobile phase without acetonitrile was recommended for the HPLC analyses. Developed SPE and HPLC methods have been applied for M, M3G, and M6G monitoring in serum samples of the cancer patient after morphine application, according to the time schedule illustrated in Table 4. High performance liquid chromatograms of SPE extracts of clinical samples M1 and M2 (a) together with standard mixture (b) are shown in Figs. 4a, b. From results in Table 4 and Figs. 4a, b, it is possible to see that concentrations of both glucuronides in patient serum samples were increasing significantly in the period of one month, M3G from about 3 mg/mL to 20 mg/mL, M6G from 0.2 mg/mL to 4.5 mg/mL. These results are in agreement with some published data, when concentrations of parent morphine in cancer patient samples have also been very low or near to the limit of determination.[5] In our case, patient sample M2 has contained 160 ng/mL, which is nearly 20 times higher than detection limit, but only about eight times higher than limit of determination of morphine in our assay (23 ng/mL). Morphine concentrations in clinical samples M1 and M3 were under the limit of determination.

In conclusion, the simple generic SPE procedure with the application of HLB sorbent, has been developed for simultaneous isolation of morphine and its

Table 3. The Influence of Washing Volume on Extraction Recoveries of Analytes ($n = 4$)

	Extraction Recovery (%)					
Volume of Water (mL)	M3G	RSD (%)	M6G	RSD (%)	M	RSD (%)
1	92.6	1.5	99.0	1.4	79.5	1.6
2	42.8	1.8	54.6	1.3	81.0	1.4
3	32.5	1.3	31.0	1.6	85.5	1.4

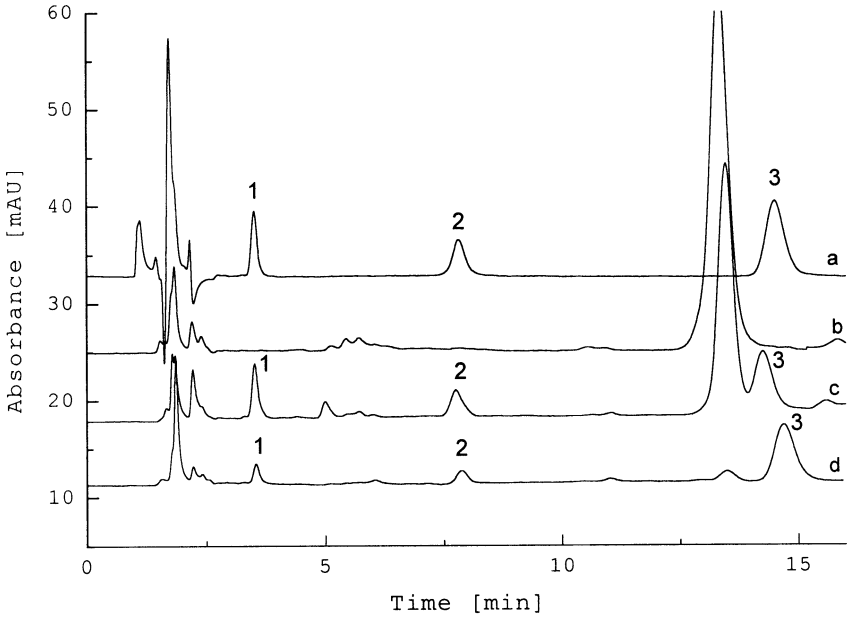

Figure 3. High performance liquid chromatogram of standard mixture of M3G, M6G, M, blank serum and spiked blank serum. High performance liquid column: Symmetry Shield RP8, mobile phase: 1% acetonitrile + 99% 20 mM Na_2HPO_4, pH 6.5; $F = 1.0$ mL/min. Detection: UV, 210 nm, injection volume: 20 μL. Separation order: 1—M3G, 2—M6G, 3—M. (a) standard mixture, (b) blank serum, washing: 1 mL water, (c, d) spiked blank serum, M: 2.02 μg/mL, M3G: 0.9 μg/mL, M6G: 1.0 μg/mL, washing: 1 mL water (c), 3 mL water (d).

Table 4. Data for Analyzed Clinical Serum Samples (M1, M2, M3) of the Cancer Patient

Sample	Date of Morphine Administration	Amount of Administrated Morphine	Determinated Concentrations (μg/mL)		
			M3G	M6G	M
M1	23/2/01	40 mg/day (infusion)	2.9	0.23	—
M2	7/3/01	3 tabs/day (200, 160, 200 mg)	11.3	1.25	0.16
M3	20/3/01	3 tabs/day (200, 160, 200 mg)	21.1	4.5	—

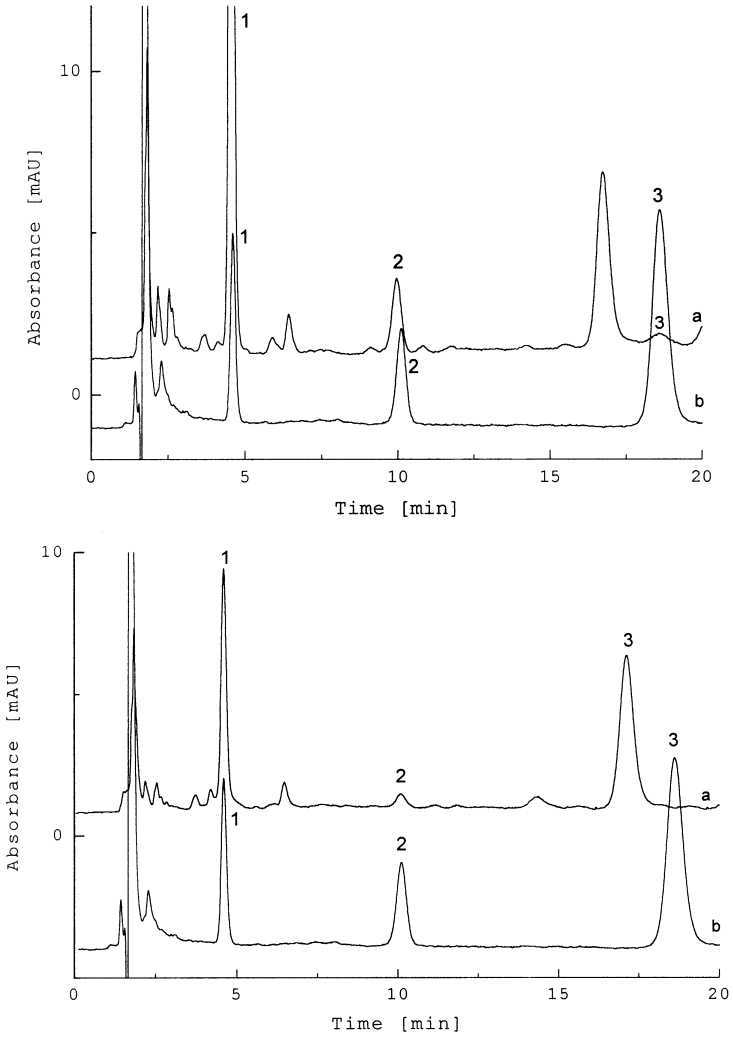

Figure 4. (a) High performance liquid chromatogram of clinical serum sample (M1) and standard mixture of M, M3G, M6G. High performance liquid column: Symmetry Shield RP8, mobile phase: 20 mM Na_2HPO_4, pH 6.5; $F = 1.0$ mL/min. Detection: UV, 210 nm, injection volume: 20 μL. Separation order: 1-M3G, 2-M6G, 3-M. (a) clinical serum sample M1, (b) standard mixture. (b) High performance liquid chromatogram of clinical serum sample (M2) and standard mixture of M, M3G, M6G. High performance liquid column: Symmetry Shield RP8, mobile phase: 20 mM Na_2HPO_4, pH 6.5; $F = 1.0$ mL/min. Detection: UV, 210 nm, injection volume: 20 μL. Separation order: 1—M3G, 2—M6G, 3—M. (a) clinical serum sample M2, (b) standard mixture.

glucuronides from serum samples, before the HPLC analysis of all analytes. The developed SPE–HPLC assay is applied at National Cancer Institute in Bratislava, Slovakia for routine monitoring M, M3G, and M6G in serum samples of cancer patients.

ACKNOWLEDGMENTS

This research was supported by the Slovak Grant Agency for Science, VEGA, Grants No. 1/6102/99 and 1/9128/02. The authors would like to thank Waters Corp. Milford, USA for a Symmetry Shield RP8 column with a guard column and Oasis SPE cartridges.

REFERENCES

1. Hoffman, M.; Xu, J.-C.; Smith, C.; Fanelli, C.; Pascal, V.; Degaetano, C.; Meenan, G.; Lehrer, M.; Lesser, M.; Citron, M. A Pharmacodynamic Study of Morphine and its Glucuronide Metabolites After Single Morphine Dosing in Cancer Patients with Pain. Cancer Invest. **1997**, *15* (6), 542–547.
2. Zuccaro, P.; Ricciarello, R.; Pichini, S.; Pacifici, R.; Altieri, I.; Pellegrini, M.; Ascenzo, G.D. Simultaneous Determination of Heroin, 6-Monoacetylmorphine, Morphine, and its Glucuronides by Liquid Chromatography-Atmospheric Pressure Ionspray-Mass Spectrometry. J. Anal. Toxicol. **1997**, *21*, 268–277.
3. Pacifici, R.; Pichini, S.; Altieri, I.; Coronna, A.; Passa, A.R.; Zuccaro, P. High-Performance Liquid Chromatographic-Electrospray Mass Spectrometric Determination of Morphine and its 3- and 6-Glucuronides: Application to Pharmacokinetic Studies. J. Chromatogr. B **1995**, *664*, 329–334.
4. Zheng, M.; McErlane, K.M.; Ong, M.C. High-Performance Liquid Chromatography-Mass Spectrometry-Mass Spectrometry Analysis of Morphine and Morphine Metabolites and its Application to a Pharmacokinetic Study in Male Sprague–Dawley Rats. J. Pharm. Biomed. Anal. **1998**, *16*, 971–980.
5. Naidong, W.; Lee, J.W.; Jiang, X.; Wehling, M.; Hulse, J.D.; Lin P.P. Simultaneous Assay of Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide in Human Plasma Using Normal-Phase Liquid Chromatography-Tandem Mass Spectrometry with a Silica Column and an Aqueous Organic Mobile Phase. J. Chromatogr. B **1999**, *735*, 255–269.

6. Blanchet, M.; Bru, G.; Guerret, M.; Bromet-Petit, M.; Bromet, N. Routine Determination of Morphine, Morphine 3-b-*D*-Glucuronide and Morphine 6-b-*D*-Glucuronide in Human Serum by Liquid Chromatography Coupled to Electrospray Mass Spectrometry. J. Chromatogr. A **1999**, *854*, 93–108.
7. Tyrefors, N.; Hyllbrant, B.; Ekman, L.; Johansson, M.; Langström, B. Determination of Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide in Human Serum by Solid-Phase Extraction and Liquid Chromatography-Mass Spectrometry with Electrospray Ionization. J. Chromatogr. A **1996**, *729*, 279–285.
8. Schänzle, G.; Li, S.; Mikus, C.; Hofmann, U. Rapid, Highly Sensitive Method for the Determination of Morphine and its Metabolites in Body Fluids by Liquid Chromatography-Mass Spectrometry. J. Chromatogr. B **1999**, *721*, 55–65.
9. Shou, W.Z.; Pelzer, M.; Addison, T.; Jiang, X.; Naidong, W. An Automatic 96-Well Solid Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry Method for the Analysis of Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide in Human Plasma. J. Pharm. Biomed. Anal. **2002**, *27*, 143–152.
10. Svensson, J.O.; Yue, Q.Y.; Säwe, J. Determination of Codeine and Metabolites in Plasma and Urine Using Ion-Pair High Performance Liquid Chromatography. J. Chromatogr. B **1995**, *674*, 49–55.
11. Bourquin, D.; Lehmann, T.; Hämmig, R.; Buhrer, M.; Brenneisen, R. High-Performance Liquid Chromatography Monitoring of Intravenously Administered Diacetylmorphine and Morphine and Their Metabolites in Human Plasma. J. Chromatogr. B **1997**, *694*, 233–238.
12. Ary, K.; Róna, K. LC Determination of Morphine and Morphine Glucuronides in Human Plasma by coulometric and UV Detection. J. Pharm. Biomed. Anal. **2001**, *26*, 179–187.
13. Meng, Q.C.; Cepeda, M.S.; Kramer, T.; Zou, H.; Matoka, D.J.; Farrar, J. High-Performance Liquid Chromatographic Determination of Morphine and its 3- and 6-Glucuronide Metabolites by Two-Step Solid-Phase Extraction. J. Chromatogr. B **2000**, *742*, 115–123.
14. Wright, A.W.E.; Smith, M.T. Improved One-Step Solid-Phase Extraction Method for Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide from Plasma and Quantitation Using High-Performance Liquid Chromatography with Electrochemical Detection. Ther. Drug Monit. **1998**, *20*, 215–218.
15. Huwyler, J.; Rufer, S.; Küsters, B.; Drewe, J. Rapid and Highly Automated Determination of Morphine and Morphine Glucuronides in Plasma by On-line Solid-Phase Extraction and Column Liquid Chromatography. J. Chromatogr. B **1995**, *674*, 57–63.

16. Beike, J.; Köhler, H.; Brinkmann, B.; Blaschke, C. Immunoaffinity Extraction of Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide from Blood of Heroin Victims for Simultaneous High-Performance Liquid Chromatographic Determination. J. Chromatogr. B **1999**, *726*, 111–119.
17. *OASIS HLB, Applications Notebook*; Waters Corp.: Milford, USA, 1998.
18. Blahová, E.; Brandšteterová, E.; Netriová, J. Stationary Phases of New Generation in HPLC Determination of Morphine and its Metabolites. Microchim. Acta **2002**, *in press.*
19. Cheng, Y.-F.; Neue, U.D.; Bean, L. Straightforward Solid-Phase Extraction Method for the Determination of Verapamil and its Metabolite in Plasma in a 96-Well Extraction Plate. J. Chromatogr. A **1998**, *828*, 273–281.

Received April 24, 2002
Accepted May 30, 2002
Manuscript 5834