Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 65 (2005) 67–73
Talanta
www.elsevier.com/locate/talanta

Liquid chromatographic high-throughput analysis of ketamine and its metabolites in human plasma using a monolithic silica column and solid phase extraction

Hassan Y. Aboul-Enein*, Mohamed M. Hefnawy

Pharmaceutical Analysis Laboratory, Biological and Medical Research Department (MBC-03), King Faisal Specialist Hospital and Research Centre, P.O. Box, 3354, Riyadh 1211, Saudi Arabia

Received 6 January 2004; received in revised form 14 April 2004; accepted 12 May 2004
Available online 14 July 2004

Abstract

A rapid and sensitive liquid chromatographic (LC) assay was developed for the simultaneous determination of ketamine (KE) and its two main metabolites, namely, norketamine (NK) and dehydronorketamine (DHNK) in human plasma. Each compound together with an internal standard (Labetalol) was extracted from the plasma matrix using solid phase extraction (SPE). The applicability of monolithic LC phases in the field of quantitative bioanalysis has been evaluated. The existing method with UV detection set at 220 nm was successfully transferred from a conventional reversed phase column to a 10 cm × 4.6 mm i.d. monolithic silica column. By simply increasing the mobile phase flow-rate, run times were about six-fold reduced and consumption of mobile phase were about two-fold decreased, while the chromatographic resolution of the analytes remain unaffected. The method was validated over the range 25–2000 ng/mL for KE, 25–1500 ng/mL for NK, and 15–750 ng/mL for DHNK. The method proved to be precise (within-run precision ranges from 2.2 to 7.2% and between-run precision ranges from 3.7 to 8.2%) and accurate (within-run accuracies ranged from 1.3 to 7.2% and between-run accuracies ranged from 1.5 to 8.7%). The mean absolute recoveries were 95.3, 96.9, and 103.9% for KE, NK and DHNK, respectively. The limit of quantitation (LOQ) and limit of detection (LOD) for KE and NK in human plasma were 25 and 12.5 ng/mL, respectively, and for DHNK were 15 and 7.5 ng/mL (S/N = 3). The assay should be suitable for use in routine determination of KE and its metabolites in human plasma.

Keywords: Liquid chromatographic high-throughput analysis; Monolithic silica; Solid phase extraction; Ketamine and its metabolites

1. Introduction

The ever increasing need for speed and efficient use of time in the pharmaceutical and other fields places a demand for the development of faster higher throughput analytical procedures. The rapid trace level quantitative determination of drugs and their metabolites remains a challenge, which is often driven by the need for same-day turnaround of results from large number of biological samples [1]. For HPLC-based assays, the process of reducing analysis time, while adequately resolving analytes from endogenous components and metabolites is often accomplished with short columns packed with small particles. The theoretical advantages for small packing particles include higher optimum linear velocities as well as shallower slopes in the high velocity region of plate height versus linear velocity curves [2].

In principle, high speed, high resolution separation can be obtained by operating small particle packed columns (e.g., $L = 50$ mm, $d = 3$ μm) at high flow-rates. Unfortunately, the high back pressure associated with these columns effectively limits their operation to mobile phase flow-rates of less than about 2 mL/min. Many researchers have been trying to overcome the problem of high pressure drop associated with the use of small particles by employing ultrahigh pressure liquid chromatography (UHPLC) [3], capillary electrochromatography (CEC) [4] or by open tube liquid chromatography (LC) [5]. Many drugs and biomedically important compounds are bases, the analysis of which remains problematic due to poor peak shapes which are often experienced in reversed phase chromatography. An excellent review of these prob-

* Corresponding author. Tel.: +966 1 442 7859; fax: +966 1 442 7858.
E-mail address: enein@kfshrc.edu.sa (H.Y. Aboul-Enein).

0039-9140/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.talanta.2004.05.021

lems was presented by Rogers and Dorsey [6]. Despite this interest, relatively little work has been done on the effects of flow-rate and temperature on the peak shape of basic compounds. Furthermore, increased temperature (up to 60 °C) may give substantial improvement in the peak shape of basic compounds [7]. Recently, monolithic silica phases have been prepared as an alternative to smaller particle-based columns. These columns made of a single piece of monolithic silica and possess a biporous structure consisting of larger macropores (2 μm) that permit high eluent flow-rates at a limited back pressure and smaller mesopores (13 nm) that provide a high surface area for high efficiency [8]. Therefore, it is possible to perform analyses with high linear flow velocity but without significantly reduced separation efficiency. The main advantage of monolithic columns, therefore is that analysis time can be decreased without compromising resolution.

The utility of monolithic silica columns for high-throughput bioanalysis in a drug discovery environment has been demonstrated [9]. The columns have been utilized to determine bexarotene in plasma and the determination of dextromethorphan and metabolites in urine [10]. Also, monolithic columns have also been used to analyze six hydroxylated debrisoquine isomers [11], ochratoxin A in different wines [12] and cocaine with its metabolites, morphine in presence of their metabolites in human plasma [13]. The monolithic column has been used to determine methylphenidate with its de-esterified metabolite in rat plasma [1] and rofecoxib with its metabolites in human plasma [2].

Ketamine (KE), chemically known as 2-(2-chlorophenyl)-2 methyl aminocyclohexanone is an anesthetic agent that has been widely used since 1970 for the induction of anesthesia. It has a rapid onset and short duration of action, so it is a preferred agent for short term surgical procedures [14]. KE possesses sedative and potent analgesic properties at sub-anesthetic doses [15]. KE produces post-hypnotic emergence reactions, such as prolonged hallucination. The frequency of KE abuse is increasing [16] and fatal KE poisoning cases have been reported [17,18]. KE undergoes an extensive liver metabolism by the hepatic microsomal cytochrome P450 enzymes *N*-demethylation to norketamine (NK) [19]. The cyclohexanone ring also undergoes oxidative metabolism to form the second metabolite dehydronorketamine (DHNK). These two metabolites, especially NK, may contribute to the pharmacological effect of KE [20]. In view of the growing importance of KE, both, as a therapeutic agent and more recently, as a drug of abuse, we reported a rapid and sensitive HPLC method for the determination of KE and its two major metabolites, namely, NK and DHNK in human plasma. Several HPLC methods for the analysis of KE and its metabolites, have been described in human plasma [21–25] and in equine serum [26]. However, the methods described require derivatization [21] or column thermostabilization [23] or time consuming and lack sensitivity for an accurate determination of KE, NK, and DHNK.

It is the aim of this paper to develop and validate a high-throughput LC method with monolithic columns for quantitative bioanalysis of KE and its major metabolites in human plasma.

2. Experimental

2.1. Materials

KE, NK, and DHNK were generously supplied by Parke-Davis Laboratories (Ann Arbor, MI, USA), whereas the internal standard labetalol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, triethylamine, reagent grade phosphoric acid were obtained from Fisher Scientific (Fairlawn, NJ, USA). Analytical reagent grade monobasic sodium phosphate, sodium hydroxide, sodium acetate, and glacial acetic acid were purchased from BDH Chemicals (Poole, UK). Water was purified with a cartridge system (Continental Water Systems, Roswell, GA, USA). Drug-free human plasma was obtained from the King Faisal Specialist Hospital and Research Centre Blood Bank.

2.2. Instrumentation

The chromatographic analysis was performed on HPLC system consisted of a Waters solvent delivery pump (Model 510, Milford, MA, USA), Waters injector with a 20 μL sample loop (Model WISP 710B), Lambda max model 481 LC spectrophotometry UV, and a Hewlett-Packard 3394A integrator (Avondale, PA, USA). Separations were performed on a reversed phase monolithic silica column (Chromolith Performance RP-18e, 100 mm × 4.6 mm i.d., Merck Kga A, Darmstadt, Germany).

2.3. Chromatographic conditions

KE, NK, DHNK, and internal standard were separated using a mobile phase consisted 30 mM monobasic sodium phosphate:acetonitrile, 75:25 (v/v). The buffer pH was adjusted to 7.2 with sodium hydroxide prior to mixing with the acetonitrile. The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon, Millipore (Yonezawa, Japan) and degassed in an ultrasonic bath for 15 min before use. The HPLC pump flow-rate was 3.0 mL/min and the injection volume was 20 μL. The chromatogram was monitored by UV detection at a wavelength of 220 nm.

2.4. Preparation of stock and standard solutions

Standard solutions of KE, NK, DHNK, and the internal standard labetalol hydrochloride were prepared in deionized water to give a concentration of 10 mg/mL. Appropriate dilutions of the individual analyte stock solution were made in deionized water to provide 10 μg/mL standard

solutions which were used for spiking human plasma. A seven point non-zero calibration standard curves ranged 25–2000 ng/mL, 25–1500 ng/mL, and 15–750 ng/mL for KE, NK, and DHNK, respectively, were prepared by spiking the drug-free plasma with appropriate volumes of KE, NK, and DHNK. The quality control (QC) samples, at three concentration levels i.e., 50, 500, 1500 ng/mL; 50, 500, 1000 ng/mL; and 25, 300, 700 ng/mL for KE, NK, and DHNK, respectively, were prepared in similar manner from the stock solution. Before the spiking, the drug-free plasma was tested to make sure that there was no endogenous interference at the retention of KE, NK, DHNK, and internal standard. The QC samples were extracted with the calibration standards to verify the integrity of the method.

2.5. Solid phase extraction (SPE)

Waters Oasis HLB and Sep-Pak C18, C8, and CN cartridges were studied. Human plasma sample (0.5 mL) was placed into 1.5 mL Eppendrof tube and accurately measured aliquots of KE, NK, and DHNK were added. Then 30 μL of the internal standard (10 ug/mL) was added to each tube and diluted with water to 1 mL and vortex vigorously for 60 s to give final concentration for KE, NK, and DHNK cited in Table 2. Cartridges were conditioned with 2 × 1 mL methanol and 2 × l mL deionized water before applying the plasma samples. Care was taken that the cartridges did not run dry. The entire spiked plasma samples were then transferred to SPE cartridges. Vacuum was then applied to obtain a flow through the cartridges of 0.5 mL/min. The cartridges were then washed with 2 × 500 μL deionized water. The cartridges were then dried under vacuums for 5 min. All SPE cartridges were eluted with 2 × 500 μL methanol containing 1% TEA. Both Sep-Pak CN and C8 showed absolute recoveries of 60–75% for the analytes and internal standard. Oasis HLB cartridge showed recoveries in excess 80%, while Sep-Pak C18 showed recoveries in excess 90%. Thus, the Sep-Pak C18 cartridges were selected for use in the assays of KE, NK, and DHNK plasma samples. The eluting solvent was evaporated to dryness using a Savant speed vac concentrator (Farmingdale, NJ, USA). The residue was dissolved in 500 μL deionized water and 20 μL was injected into an HPLC system.

2.6. Selectivity

The selectivity of the assay was checked by analyzing four independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analytes.

2.7. Linearity

Calibration plots for the analytes in plasma were prepared by diluting stock solutions with pooled human plasma to yield seven concentrations over the range of 25–2000 ng/mL, 25–1500 ng/mL, and 15–750 ng/mL for KE, NK, and DHNK, respectively. Calibration standards at each concentration were extracted and analyzed in triplicate. Calibration curves of KE and the selected metabolites were constructed using the observed analyte peak area over internal standard peak area vs. nominal concentrations of the analytes. Least squares linear regression analysis of the data gave slope, intercept and correlation coefficient data. From this data, a first order polymonial model was selected for each analyte.

2.8. Precision and accuracy

The within-run and between-run accuracy and precision of the assays in plasma were determined by assaying three QC samples in triplicate over a period of 3 days. The concentrations represented the entire range of the calibration curves. The lowest level was at twice the expected limit of quantitation (LOQ) for each analyte. The second level was within 10 times of LOQ and third level was at 75% of the upper concentration of the calibration curves. Calibration curves were prepared and analyzed daily and linear models were used to determine concentrations in the QC samples. The nine measured concentrations per concentration level (triplicates from three runs) were subjected to estimate the within-run and between-run precision. Precision was reported as % relative standard deviation (%R.S.D.) = (S.D./mean) × 100. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Percent accuracy was reported as %error = ((nominal concentration−measured concentration)/nominal concentration) × 100.

2.9. Limit of detection and LOQ

The limit of detection (LOD) and the LOQ were determined as 3 and 10 times the baseline noise, respectively, following the United States Pharmacopoeia [27]. The results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear squares treatment of the results along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of the residuals ($S_{y/x}$) was shown in Table 3. The good linearity of the calibration graphs and the negligible scatter of experimental points are clearly evident by the values of the correlation coefficient and standard deviation [28]. The robustness of the method is demonstrated by the versatility of the experimental factors that affect the peak area.

2.10. Recovery

The absolute recoveries of each analyte from plasma was calculated by comparing drug peak area of the spiked analyte samples to unextracted analyte of stock solution that has been injected directly into an HPLC system. The assay absolute recovery for each compound, at each concentra-

tion, was computed using the following equation: absolute recovery = (peak area of extract/mean peak area of direct injection) × 100.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The chemical structures for KE and its two main metabolites, namely, NK and DHNK are shown in Fig. 1. The LC method carried out in this study aimed at developing a high-throughput chromatographic system capable of eluting and resolving KE, NK, and DHNK from human plasma, low ng/mL sensitivity and a simple extraction method that could be easily automated. The preliminary investigations were directed toward the effect of various variables on the system suitability of the method. The parameters assessed include the type and percentage of the organic modifier, the type and concentration of buffer and the pH of the mobile phase. The more hydrophilic of DHNK and NK tend to elute first, whereas KE tended to elute much later in the run. Generally, the retention of KE was significantly influenced by the pH of the mobile phase in the range 6.0–7.5, whereas the retention of the two metabolites is less affected. The concentration of the organic modifier (acetonitrile) strongly influenced the retention behavior of the three compounds. Ultimately, it was possible to separate KE and the selected metabolites, using a mobile phase consisting of 75:25 (v/v), 30 mM sodium phosphate monobasic monohydrate-acetonitrile. The buffer pH was adjusted to 7.2 with sodium hydroxide prior to mixing with the acetonitrile (Table 1). These conditions were found to give good selectivity and sensitivity in 4-min run.

Table 1
Analytical figures of merit for ketamine and its metabolites

Analyte	k	Tailing factor[a]	Rs[b]	α[c]
Ketamine	22.5	1.1	6.3	1.7
Norketamine	13.0	1.0	3.5	1.3
Dehydronorketamine	10.0	1.0	2.5	1.2

[a] Calculated at 5% peak height.
[b] $Rs = 2\,(t_2 - t_1)/(w_{b2} + w_{b1})$, where t_2 and t_1 are the retention of the second and first peaks and w_{b2} and w_{b1} are the half peak width of the second and first peaks.
[c] Separation factor, calculated as k_2/k_1.

3.2. Applications to spiked human plasma

In the course of developing a SPE procedure for plasma sample clean up, several types of cartridges were investigated (Water Oasis HLB and Sep-Pak C18, C8, and cyanopropyl). The cyanopropyl cartridge showed interfer-

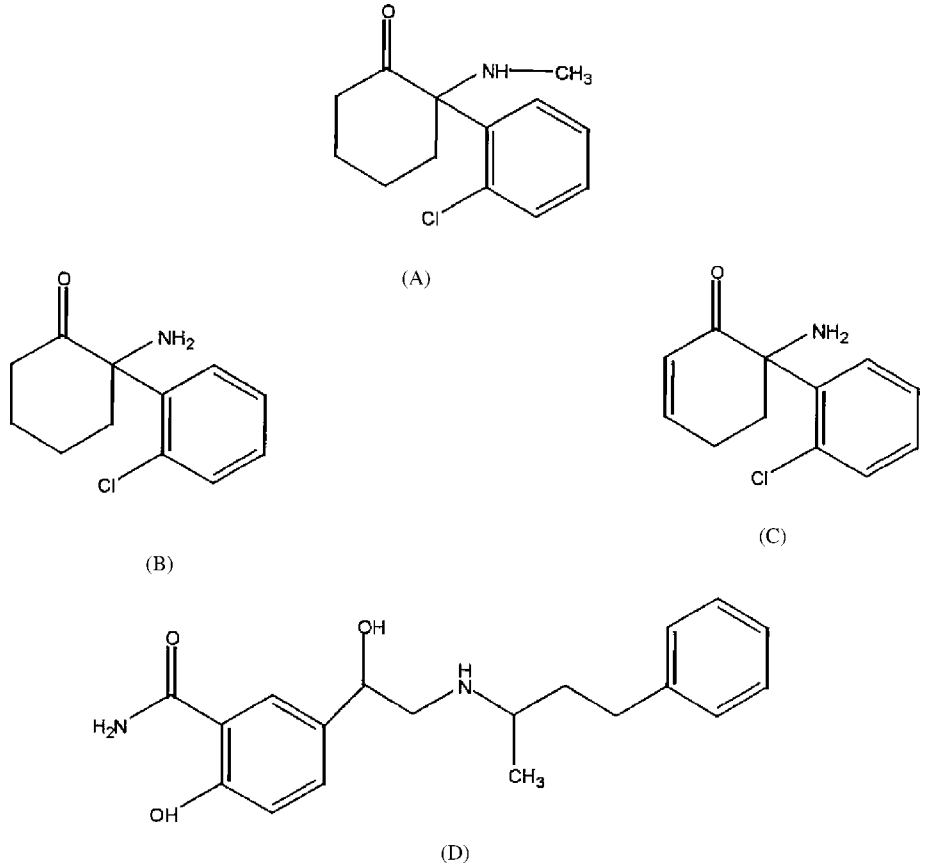

Fig. 1. The chemical structure of (A) ketamine, (B) norketamine, (C) dehydronorketamine, and (D) labetalol (IS).

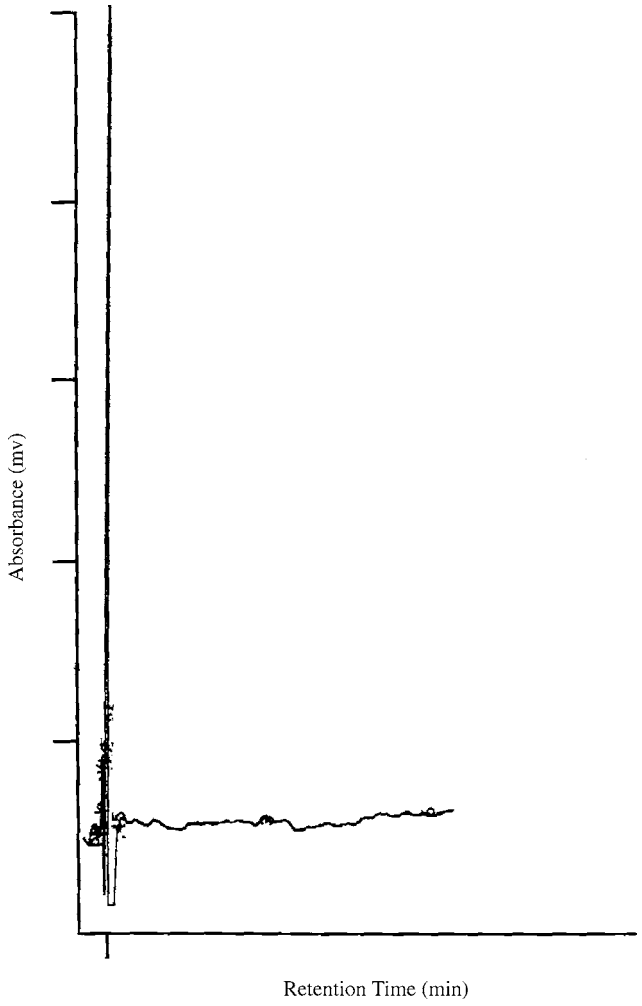

Fig. 2. Chromatogram of blank pooled human plasma.

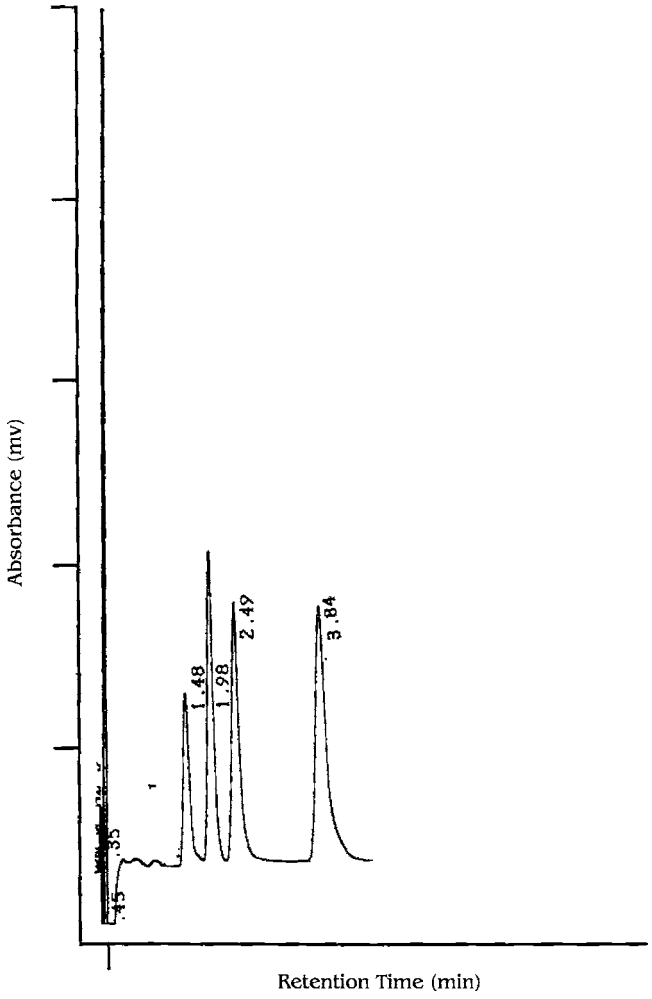

Fig. 3. Chromatogram of pooled human plasma spiked with 500 ng/mL KE (3.84 min), 500 ng/mL NK (2.49 min), 300 ng/mL DHNK (1.98 min) and 300 ng/mL labetalol (1.48 min).

ence endogenous plasma peaks at retention time of internal standard and DHNK. An octyl (C8) SPE column was also found to be unacceptable due to low recoveries for KE, NK, and DHNK. Oasis HLB cartridge showed recoveries in excess 80%, whereas an octadecyl (C18) SPE column gave high recoveries for KE and its metabolites (more than 90%), while at the same time removing endogenous interference. Figs. 2 and 3 show chromatograms of a blank plasma sample and a spiked human plasma, respectively.

3.3. Precision and accuracy

A summary of the accuracy and precision results is given in Table 2. The acceptance criteria (within-run and between-run %R.S.D. < 15% and accuracy between 85 and 115%) were met in all cases. The precision and accuracy of the method were determined by using plasma samples spiked at three levels (Table 2). The data indicate that within-run precision ranged from 2.2 to 7.2% ($n = 3$) and within-run accuracy ranged from 1.3 to 7.2% ($n = 3$) for the three compounds. The between-run precision ranged from 3.7 to 8.2% ($n = 9$) and between-run accuracy ranged from 1.5 to 8.7% ($n = 9$) for the three analytes.

3.4. Linearity

The calibration curves showed good linearity in the range 25–2000 ng/mL for KE, 25–1500 ng/mL for NK and 15–750 ng/mL for DHNK (Table 3). The correlation coefficients (R^2) of calibration curves of each drug were higher than 0.999 as determined by least squares analysis.

3.5. LOD and LOQ

The LOD, as defined in Section 2 were12.5 ng/mL for KE and NK and 7.5 ng/mL for DHNK (Table 3). The LOQ of each calibration graph was 25 ng/mL for KE, NK, and 15 ng/mL for DHNK. Table 3 also shows the results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the least squares treatment of the results along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of the residuals ($S_{y/x}$).

Table 2
Accuracy and precision data for the analysis of ketamine and its metabolites in human plasma

Analyte	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Recovery (%)	Error (%)	R.S.D. (%)
Within-run[a]					
Ketamine	50	46.95 ± 2.39	93.9	6.1	5.1
	500	476.65 ± 14.45	95.3	4.7	3.0
	1500	1449.75 ± 38.65	96.6	3.4	2.7
Norketamine	50	47.35 ± 1.85	94.7	5.3	3.9
	500	487.85 ± 10.82	97.5	2.4	2.2
	1000	986.76 ± 21.69	98.7	1.3	2.2
Dehydronorketamine	25	25.71 ± 1.85	102.8	7.2	7.2
	300	316.25 ± 9.85	105.4	5.4	3.1
	700	724.25 ± 22.81	103.5	3.5	3.2
Between-run[b]					
Ketamine	50	47.75 ± 2.35	95.5	4.5	4.8
	500	456.65 ± 19.95	91.3	8.7	4.4
	1500	1393.45 ± 58.56	92.9	7.1	4.2
Norketamine	50	48.95 ± 2.95	97.9	2.1	6.0
	500	492.35 ± 19.45	98.5	1.5	3.9
	1000	1023.59 ± 37.93	102.34	2.4	3.7
Dehydronorketamine	25	26.23 ± 2.15	104.9	4.9	8.2
	300	320.55 ± 21.75	106.8	6.8	6.8
	700	737.95 ± 48.50	105.3	5.3	7.5

[a] Mean ± S.D. based on $n = 3$.
[b] Mean ± S.D. based on $n = 9$.

Table 3
Validation parameters for the determination of ketamine and its metabolites using the proposed method

Parameters	Ketamine	Norketamine	Dehydronorketamine
Concentration range (ng/mL)	25–2000	25–1500	15–750
Intercept (A)	0.0773	0.0564	0.0817
Slope (B)	0.0077	0.0102	0.0228
Correlation coefficient (R^2)	0.9995	0.9993	0.9994
$S_{y/x}$	0.0528	0.0368	0.0195
S_a	0.0029	0.0045	0.0018
S_b	0.0004	0.0006	0.0002
LOQ (ng/mL)	25.0	25.0	15.0
LOD (ng/mL)[a]	12.5	12.5	7.5

[a] S/N = 3.

3.6. Selectivity

The analytical figures of merit for this method are shown in Table 1. KE, NK, and DHNK were well separated under the LC conditions applied. Retention times were 1.98, 2.49, and 3.84 min for KE, NK, and DHNK, respectively. No interference was observed in drug-free human plasma samples. Figs. 2 and 3 show chromatograms of a blank plasma sample and a calibration sample, respectively.

4. Conclusions

The utility of monolithic silica LC column for the determination of KE, NK, and DHNK in human plasma has been demonstrated. Monolithic LC columns are a useful means of increasing the separation efficiency per unit time of chromatographic methods, which can very simply be achieved by increasing the mobile phase flow-rate. In conventional LC, the increased efficiency per unit time can routinely be applied to reduce chromatographic run times at least three-fold, while maintaining the resolution between analyte peaks. Although, the applicability may obviously vary from one method to the other, the results shown here support the idea that most chromatographic assays on standard reversed phase columns can be rather easily transferred to commercially available monolithic LC phases. The fact that consumption of mobile phase per unit time is much higher than for conventional methods is compensated for by the much shorter run times of each analysis, which means that the total solvent consumption per analysis is comparable with that of conventional LC methods.

Acknowledgements

The authors would like to thank the King Faisal Specialist Hospital and Research Centre administration for their sup-

port for the Pharmaceutical Analysis Laboratory Research Program.

References

[1] N. Barbarin, D.B. Mawhinney, R. Black, J. Henion, J. Chromatogr. B 783 (2003) 73.
[2] P.T. Vallano, R.S. Mazenko, E.J. Woolf, B.K. Matuszewski, J. Chromatogr. B 779 (2002) 249.
[3] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Anal. Chem. 69 (1997) 983.
[4] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[5] P.P.H. Tock, C. Boshoven, H. Poppe, J.C. Kraak, J. Chromatogr. 479 (1990) 200.
[6] S.D. Rogers, J.G. Dorsey, J. Chromatogr. A 892 (2000) 57.
[7] D.V. McCalley, J. Chromatogr. A 902 (2002) 311.
[8] K. Cabrera, D. Lubda, H.M. Eggenweiller, H. Minakuchi, K. Nakanishi, J. High. Resolut. Chromatogr. 23 (2000) 93.
[9] J.T. Wu, H. Zeng, Y.Z. Deng, S.E. Unger, Rapid Commun. Mass Spectrom. 15 (2001) 1113.
[10] N.C. van de Merbel, H. Poelman, J. Pharm. Biomed. Anal. 33 (2003) 495.
[11] G. Dear, R. Plumb, D. Mallett, Rapid Commun. Mass. Spectrom. 15 (2001) 152.
[12] P. Zoellner, A. Leitner, D. Lubda, K. Cabrera, W. Lindner, Chromatographia 52 (2000) 818.
[13] W.V. Caufield, J.T. Stewart, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 2977.
[14] Y. Hijazi, M. Bolon, R. Boulieu, Clin. Chem. 47 (2001) 1713.
[15] M.S. Sadove, M. Shulman, S. Haloro, N. Fevold, Anesth. Anal. Curr. Res. 50 (1971) 452.
[16] J. Lawrence, Microgram 29 (1991) 202.
[17] M. Licata, G. Pierini, J. Forensic Sci. 39 (1994) 1314.
[18] K.A. Moore, E.M. Kilbane, R. Jones, G.W. Kunsman, B. Levine, M. Smith, J. Forensic Sci. 2 (1997) 1183.
[19] P.F. White, W.L. Way, A.J. Trevor, Anesthesiology 56 (1982) 119.
[20] D.L. Reich, G. Silvay, Can. J. Anaesth. 36 (1989) 186.
[21] L.L. Needham, M.M. Kochhanr, J. Chromatogr. 114 (1975) 220.
[22] G. Geisslinger, S. Menzel-Soglowek, H.D. Kamp, K. Brune, J. Chromatogr. Biomed. Appl. 106 (1991) 165.
[23] J.O. Svensson, L.L. Gustafsson, J. Chromatogr. B Biomed. Appl. 678 (1996) 373.
[24] S. Bolze, R. Boulieu, Clin. Chem. 44 (1998) 560.
[25] Y. Yanagihara, M. Ohtani, S. Kariya, K. Uchino, T. Aoyama, Y. Yamamura, T. Iga, J. Chromatogr. B Biomed. Appl. 746 (2000) 227.
[26] S.S. Seay, D.P. Aucoin, K.L. Tyczkowska, J. Chromatogr. Biomed. Appl. 131 (1993) 281.
[27] The United States Pharmacopeia, 24th ed., United States Pharmacopeial Convention, Rockville, MD, USA, 2000, pp. 2150.
[28] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, Wiley, New York, 1984.