Journal of Chromatography B, 876 (2008) 170–174

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of volatile biomarkers for apoptosis and necrosis by solid-phase microextraction–gas chromatography/mass spectrometry: A pharmacometabolomic approach to cisplatin's cytotoxicity to human lung cancer cell lines

Jae Sung Pyo, Hyun Kyoung Ju, Jeong Hill Park, Sung Won Kwon*

Research Institute of Pharmaceutical Sciences and College of Pharmacy, Seoul National University, Seoul 151-742, South Korea

ARTICLE INFO

Article history:
Received 5 June 2008
Accepted 17 October 2008
Available online 1 November 2008

Keywords:
Cisplatin
SPME
GC/MS
Volatile organic compounds
Biomarkers
Apoptosis
Necrosis

ABSTRACT

In order to find potential new biomarkers of cisplatin-induced apoptosis and necrosis, volatile organic compounds (VOCs) from cisplatin-treated human lung cancer cell lines were investigated. The biological system employed was human non-small cell lung carcinoma A549 cell lines. The cell lines were treated with two different concentrations of cisplatin, 100 μM and 400 μM, and apoptosis and necrosis were determined by flow cytometric analysis. For each drug concentration, the VOCs from the treated cell lines were extracted by solid-phase microextraction (SPME), and subsequently analyzed by gas chromatography/mass spectrometry (GC/MS). The compounds that change during cisplatin-induced apoptosis and necrosis of lung cancer cell lines can serve as new biomarkers. The pharmacometabolomic approach presented in this study, significantly, implicates a non-destructive, sample-thrifty and time-saving tool for finding new biomarkers for the assessment of drug-induced cell death pathways.

1. Introduction

Cis-diamminedichloroplatinum, popularly known as cisplatin, is one of the chemotherapeutic agents widely used in treatment of solid tumors associated with cervical, ovarian, testis, head and neck, endometrium and lung cancers. It has also been applied for many years to various *in vivo* and *in vitro* experiments.

However, cisplatin does possess a cytotoxic effect, and the mechanism of its cytotoxicity is well investigated [1,2]. The cisplatin concentration determines the mode of cell death [3]. Cisplatin-induced apoptosis is usually accompanied by characteristic morphological and biochemical changes such as cell shrinkage, DNA fragmentation, and chromatin condensation [3,4]. In contrast, necrosis is characterized by the loss of plasma membrane integrity, and cytosolic swelling can also be observed. It reacts covalently with the base in DNA to prevent cell division, and interferes with the calcium transduction channel. Some cytotoxic mechanisms are mediated also by reactive oxygen species (ROS), such as the superoxide, hydrogen peroxide and hydroxyl radicals produced during chemotherapeutic treatment. ROS-induced oxidative stress causes lipid peroxidation of cell membranes, which leads to generation of volatile organic compounds (VOCs) including alkanes and methylalkanes [5].

Flow cytometric measurement with annexin-V and propidium iodide double-staining procedure is the most standardized method of determining apoptosis and necrosis. However, trypsinization and damage due to cell handling before flow cytometric analysis can result in some cell death. Incubation with propidium iodide, moreover, can be harmful to cells. Furthermore, trypsinized cells cannot be reused in other experiments. Also, the stainer kits usually are expensive. Therefore, ideal *in vitro* cellular determination of the cell death pathway should be not only simple, fast, and sensitive, but also non-toxic, non-destructive, and cost-effective.

Gas chromatography/mass spectrometry (GC/MS) can conveniently be employed to analyze VOCs derived from cell metabolites such as alcohols, esters, ketones, methylalkanes and alkanes. Pauling et al. analyzed, by GC, the VOCs contained in human breath, and identified hundreds of VOCs in the exhaled gas [6]. O'Neill et al. identified 28 biomarkers, including benzene and alkane derivatives, in the exhaled gasses of lung cancer patients [7,8]. VOCs in the blood from lung cancer patients [9] and liver cancer patients [10] were also analyzed by solid-phase microextraction (SPME) and capillary GC/MS. Recently, VOCs from human colon cell line metabolites were analyzed using SPME-coupled GC/MS [11]. The chromato-

* Corresponding author. Tel.: +82 2 880 7844 fax: +82 2 886 7844.
E-mail address: swkwon@snu.ac.kr (S.W. Kwon).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.031

graphic patterns of some VOCs obtained from normal cells and cancer cells were found to be considerably different, suggesting the potential use of the identified compounds as new volatile biomarkers. In practice, VOCs have been employed by several researchers as biomarkers in the study of diverse diseases and environmental exposure [5,9,12].

The main objective of the present study, thus, was to analyze VOCs in order to discover new biomarkers for the determination of cisplatin-induced cell death. In this paper we present the first report of the development of an SPME–GC/MS method for the analysis of VOCs generated from cisplatin-treated human lung carcinoma cell lines. The experimental results suggest that the developed SPME GC/MS method has the potential to serve as a rapid, simple, and economic tool for the determination of types of cell death.

2. Experimental

2.1. Materials

Dulbecco's modified eagle medium (DMEM) Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Cisplatin and 1,3-bis(1,1-dimethylethyl)-benzene were acquired from Sigma (St. Louis, MO, USA). Decane, nonanal and 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione were obtained from Supelco (St. Louis, MO, USA).

2.2. Cell cultures

Human non-small-cell lung carcinoma A549 cell lines, obtained from Korean Cell Line Bank (KCLB®, Seoul, Korea), were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 50 $\mu g\,ml^{-1}$ of streptomycin and 50 units ml^{-1} of penicillin G in a cell-culture flask (Nunclon Delta Surface, Denmark). They were grown under standard conditions, in a 37 °C humidified incubator containing 5% CO_2. The medium was changed 2–3 times per week.

2.3. Drug treatment and cell viability measurement

To determine the effect of cisplatin on the viability of the A549 cell lines, the cells were incubated with various concentrations of cisplatin (ranging from 5 to 400 μM), and the percent cell viability was measured by methylthiazolelyldiphenyl-tetrazolium bromide (MTT) assay after 24 h of incubation. Prior to the drug treatment, the A549 cell lines were loaded into a 96-well plate (3×10^3 cells/well) and incubated at 37 °C. After 24 h of incubation for stabilization, the cisplatin solutions were added to the cell lines and incubated at 37 °C for another 24 h. Subsequently, 20 μL of MTT (5 mg/ml) solution was added to each well, and further incubated for 2 h. Finally, the medium was discarded and 100 μL of DMSO was added to each well, after which the plate was gently shaken for 5 min at room temperature. The absorbance was read at 570 nm on a SpectraMax 340 PC microplate reader (Molecular Devices; Sunnyvale, CA, USA). Cell lines not exposed to cisplatin, but grown under the same conditions as the drug-treated cells, were treated as a negative control. The cell viability percentage was calculated as follows (Test optical density/Non-treated optical density) × 100. Quadruplicate wells were applied to each concentration.

2.4. Flow cytometric analysis of cisplatin-treated A549 cell lines

In order to investigate the cytotoxicity of the cisplatin-treated A549 cell lines, a flow cytometric assay was performed using the annexin-V (A) and propidium iodide (P) double-staining method, which enables the quantification of viable, apoptotic and necrotic cells. Propidium iodide and fluorescein isothiocyanate (FITC)-labeled annexin-V (Annexin-V-fluos Staining Kit, Roche Diagnostics, Penzberg, Germany) were used to detect phosphatidylserine (PS) expression as an endpoint indicator of early apoptosis. After 24 h of incubation, the cisplatin-treated HL-60 cells (1×10^6) were collected, washed twice with phosphate-buffered saline (PBS), and centrifuged at 270 *g* for 3 min. The pellets were reconstituted in HEPES buffer (Roche Diagnostics) to a final concentration of 10^6 cells/ml. FITC-labeled annexin V (5 μl) and P (5 μl) were added to 490 μl of cell suspension, and mixed gently. After 10 min of incubation at room temperature in darkness, the cells were subjected to flow cytometric analysis (FACSCalibur, BD Bioscience; San Jose, CA, USA). Whereas the annexin V-FITC-positive and P-negative (A+/P−) cells were considered apoptotic, annexin V-FITC-positive and P-positive (A+/P+) cells together were categorized as necrotic cells. The cells not exposed to cisplatin, but grown under the same conditions as the drug-treated cells, were treated as a negative control, that is, considered to be both Annexin V-FITC-negative and P-negative (A−/P−).

2.5. Volatile organic compounds (VOCs)

In order to absorb VOCs, a manual SPME holder with polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (65 μm without epoxy resin; Supelco) was kept in the atmosphere of the cell-culture flask for 40 min at 250 °C. Before the VOC sampling, the fiber was activated in a GC oven at 250 °C for 30 min. After the VOC sampling, the holder containing the fiber was directly transferred to a GC injector. The analytes were desorbed with helium as a carrier gas (flow rate; 0.8 ml/min) for 5 min at 200 °C. During the desorption, the front part of the GC column was soaked in liquid nitrogen for fixation of desorbed VOCs. After desorption, the liquid nitrogen was removed and GC/MS measurement was started. VOC sampling was subjected to triplicate analysis for reproducibility.

2.6. GC/MS measurements

An HP 6890 Series Plus gas chromatograph (Agilent Technologies; Milan, Italy) equipped with a JMS-GC mate mass spectrometer (Jeol; Tokyo, Japan) was used for a GC/MS analysis. Helium, at a flow rate of 0.8 ml/min, was employed as the carrier gas. The gas chromatograph was operated in splitless mode with a PTV injector (Agilent Technologies) maintained at a temperature of 140 °C. Chromatographic separation was performed on a HP-5 capillary column (length 30 m, I.D. 0.32 mm, film thickness 0.25 μm, Agilent Technologies). The column temperature was maintained at 40 °C for 5 min and increased to 200 °C at the rate of 10 °C/min. Then 200 °C was maintained for 5 min. The temperatures of the ion source and the transfer line were 180 °C and 230 °C, respectively. Electron impact mass spectra were recorded at an ionization energy of 70 eV and with a cycle time of 2 scans per second. The VOCs were identified by comparison of the chromatographic retention time and the mass spectra of standards. All of the samples were measured in triplicate.

2.7. Method validation

The chromatographic standards [decane, nonanal, 1,3-bis(1,1-dimethylethyl)-benzene, and 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione] identified as increased VOCs in cisplatin-treated cell lines were used for method validation. The mixture of standard solution was prepared in methanol and SPME fiber was employed with the same condition of VOCs detection in cell lines. The linearity was calculated from the regression analysis

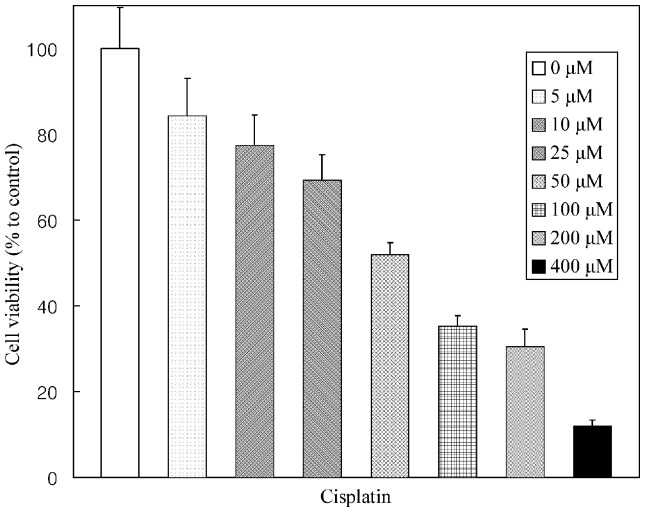

Fig. 1. Cytotoxic effects of cisplatin on A549 cell lines. After the cells were treated with 0–400 μM of cisplatin for 24 h, the viability of the cells was measured using an MTT colormetric assay.

between peak area and the analyte concentration. The accuracy was estimated by triplicate analysis of VOCs of chromatographic standards at a concentration of 10 ng/ml and calculated as follows (C_F = experimental amount of VOCs, C_A = spiked amount of VOCs)

$$\text{Percentage recovery}\,(\%) = \frac{C_F}{C_A} \times 100$$

The precision assay was evaluated with the relative standard deviation (RSD) of three measurements and calculated. RSD (%) = CV% = $\sigma \times 100/X$ (X = average, CV = coefficient of variation, σ = standard deviation).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to ICH guideline [13].

3. Results and discussion

3.1. Cytotoxic effects of cisplatin

In order to determine the optimal concentration of cisplatin for the screening of volatile compounds, the A549 cell lines were incubated with different concentrations of the drug, and the cell viability was measured by MTT assay. Fig. 1 shows the anti-neoplastic activity of cisplatin on the A549 cell lines, as well as the cell viabilities detected by MTT assay after 24 h of drug-incubation, plotted as a function of cisplatin concentration. The cell viability percentage decreased from 84% to 12% as the concentration of the drug increased from 5 μM to 400 μM.

3.2. Flow cytometric analysis

Cisplatin induces cell death in a concentration-dependent manner [14]. For better understanding of the molecular mechanisms of cisplatin-induced cell death at different concentrations, further flow cytometry investigations were carried out. The two different types of cell death, apoptosis and necrosis, have special morphological and biochemical characteristics. The early manifestations of the apoptotic cells include cell shrinkage, DNA fragmentation and chromatin condensation. By contrast, necrotic cells are characterized by early loss of plasma membrane integrity and cytosolic swelling. The onset of the safe mode of cellular destruction (apoptosis) in the A549 cell lines was visualized by monitoring the phosphatidylserine (PS) externalization, an early marker of apoptosis, by means of a cytometric analysis. A fluorochrome-labeled antibody (annexin V–FITC) to PS was used to monitor the endpoint of early apoptosis. Annexin V, a human protein with a molecular weight of 36,000, has a high affinity for PS on the cell membrane.

However, necrotic cells also release PS, as a function of the loss of cell membrane integrity. Therefore, apoptotic cells must be differentiated from those necrotic cells. A DNA staining dye (propidium iodide) used for dye exclusion allows the distinction of necrotic cells from the annexin-V-stained cell cluster. Four different concentrations (6.5, 25, 100, 400 μM) of cisplatin were treated to the A549 cell lines for the purposes of the flow cytometric analysis. Cells treated with 6.5 and 25 μM of cisplatin showed a result similar to that of the control cell lines (the data not shown). Therefore, we selected 100 and 400 μM cisplatin-treated cells to observe the apoptotic and necrotic cells.

Fig. 2 shows the dot plots recorded, after 24 h, for two different concentrations (100 and 400 μM) of cisplatin. The lower-right quadrant of the dot plot represents the early apoptotic cells with phophatidylserine externalization (annexin V-FITC-positive), and the upper-right quadrant represents the propidium iodide (P)-positive dead cell (necrotic) population. The control cell population, which was negative for both P and annexin V-FITC, is represented in the lower-left quadrant. A few apoptotic (5.09%) and necrotic (1.56%) cells can be seen in the control cells that were not subjected to cisplatin treatment (Fig. 2). However, when those cells were treated with cisplatin, the percentage of apoptotic cells increased to 18.30% (for 100 μM of the cisplatin) and 48.55% (400 μM of cisplatin). Interestingly, the percentage of necrotic cells (1.56%; control

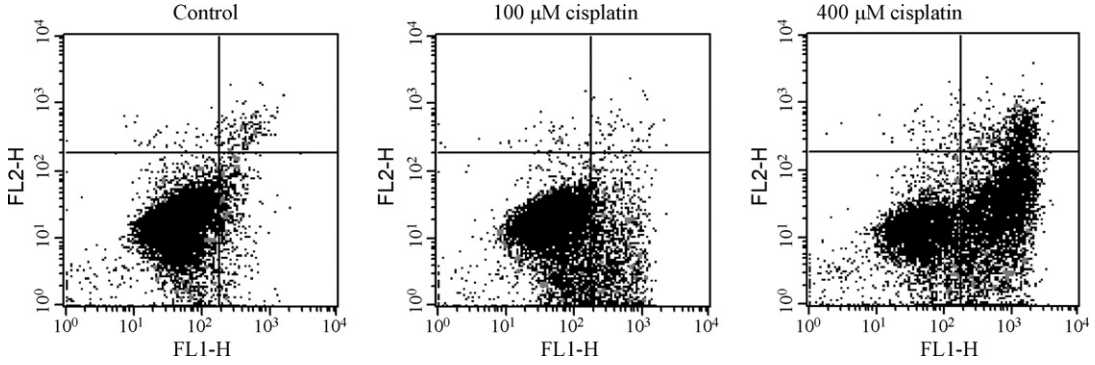

Fig. 2. Results of flow cytometry analysis of A549 cell lines treated without or with cisplatin. A dual parameter (FL1, Annexin-V; FL2, propidium iodide) was applied. The cell cluster (lower left; A–, P–) represents living cells. The cells in the lower-right area are apoptotic cells (A+, P–). The upper-right cell cluster (A+, P+) comprises necrotic cells.

Table 1
The linearity, precision, accuracy, LOD, and LOQ values of volatile organic compounds.

Peak no.	Calibration range (ppb)	R^2 (square of correlation coefficient)	Precision (%)	Accuracy (%)	LOD (ppb)	LOQ (ppb)
1	500.00–5.00	0.9998	1.02	114.78 ± 3.03	0.23	0.69
2	50.00–0.50	0.9996	8.23	94.50 ± 8.04	0.40	1.20
3	100.00–1.00	0.9999	2.56	98.25 ± 2.56	0.04	0.13
4	10.00–0.10	0.9999	7.36	105.53 ± 7.31	0.08	0.25

Table 2
Determination of VOCs in A549 cell lines characterized as apoptosis and necrosis by GC/MS with SPME.

Peak no.	Retention time (min)	Compound name	MW
1	7.68	Decane	142
2	14.15	Nonanal	142
3	18.17	1,3-bis(1,1-dimethylethyl)-benzene	190
4	24.70	2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	220

cells, 1.65%; 100 μM of cisplatin-treated cells) increased only in 400 μM of cisplatin-treated cells (11.49%). These results indicated that cisplatin might induce dose-dependent induction of apoptosis in A549 cell lines, with a subsequent shift in the necrotic phase above a specific drug concentration.

3.3. Method validation

The linearity, accuracy, precision, LOD, and LOQ were calculated using the chromatographic standards. The linearity, an important characteristic for quantitative analysis, was presented as the corre-

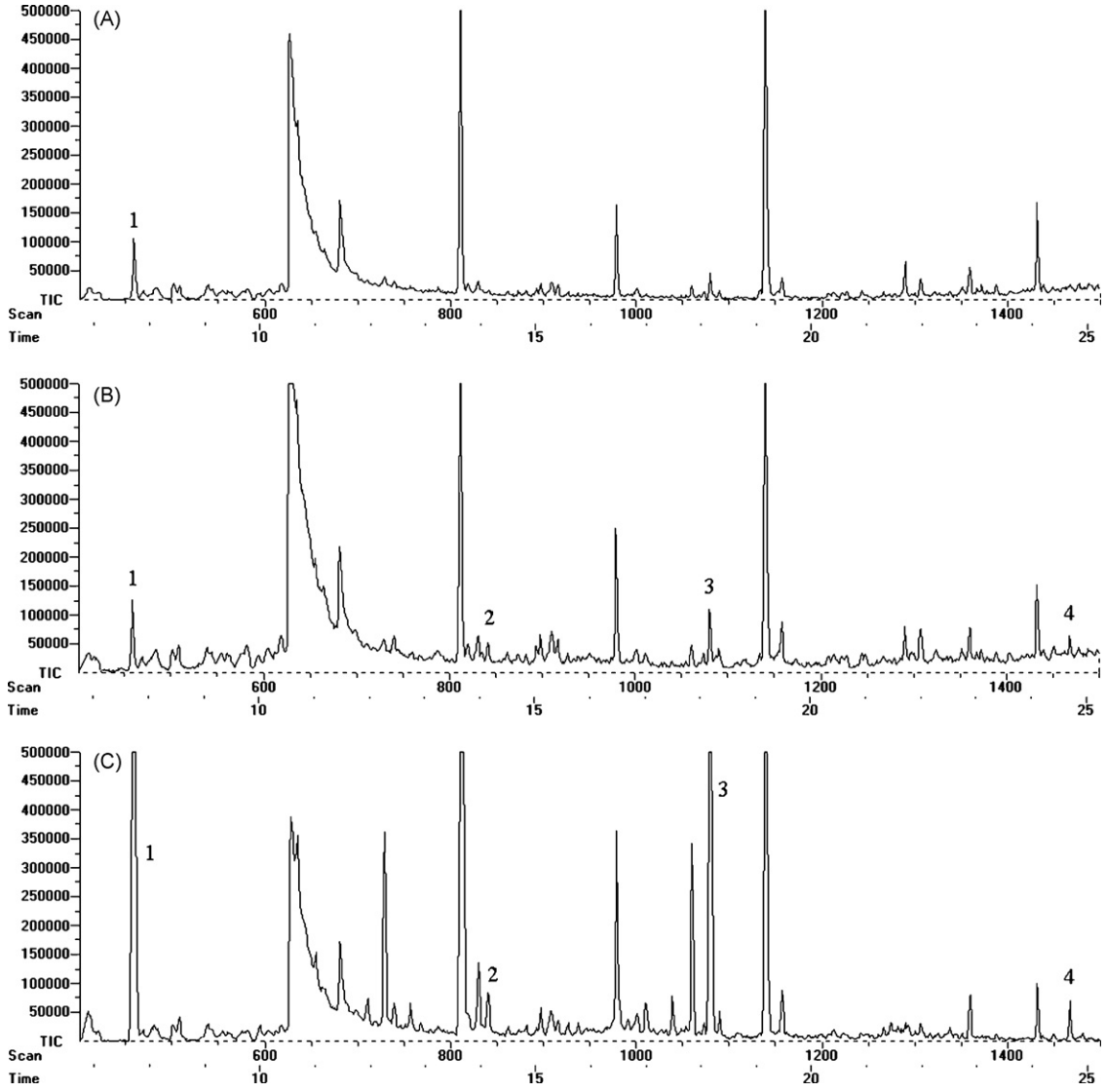

Fig. 3. Total ion chromatograms of VOCs in A549 cell lines. (A) Control cells; (B) 100 μM cisplatin-treated cells; (C) 400 μM cisplatin-treated cells (1: decane; 2: nonanal; 3: 1,3-bis(1,1-dimethylethyl)-benzene; 4: 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione).

Table 3
The peak area of volatile organic compounds.

Peak no.	Peak area (0 μM cisplatin)	Peak area (100 μM cisplatin)	Peak area (400 μM cisplatin)	R^2
1	300,494 ± 16,500	242,041 ± 12,790	9,223,601 ± 682,200	Non-available
2	24,869 ± 5,700	112,511 ± 9,862	272,401 ± 32,970	0.9969
3	99,242 ± 15,970	1,323,649 ± 227,600	6,139,116 ± 521,500	0.9884
4	29,432 ± 5,615	77,327 ± 3,872	202,428 ± 24,470	0.9991

lation coefficients (R^2) varying from 0.9996 to 0.9999. The accuracy was evaluated by analyzing triplicate of VOCs of chromatographic standards at a point of the calibration range. The range of accuracy was from 94.50 ± 8.04 (nonanal) to 114.78 ± 3.03% (decane). The precision was calculated using the standard deviation and average of VOCs peak area and all of the precisions are varying from 1.02 to 8.23%. The LOD and LOQ of the VOCs are shown in Table 1. The lowest LOD (0.04 ng/ml) and LOQ (0.13 ng/ml) were obtained from 1,3-bis(1,1-dimethylethyl)-benzene. The LODs were varying from 0.04 to 0.40 ng/ml and the LOQs were on the level from 0.13 to 1.20 ng/ml (Table 1).

3.4. Differences in volatile products during drug treatment

The formation of DNA adducts by damage-response proteins, leading to cell cycle arrest, is the major process of cisplatin cytotoxicity. The other cytotoxic mechanism is related to ROS-induced oxidative stress, which causes lipid peroxidation of polyunsaturated fatty acids in membranes, producing alkanes and methylalkanes. We found several changed alkanes and methylalkanes generated by oxidative stress in the cell headspace, as characterized by apoptosis and necrosis.

Among the changed VOCs (Fig. 3), the three compounds listed in Table 2, nonanal, 1,3-bis(1,1-dimethylethyl)-benzene, and 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione, can be used as biomarkers of apoptosis. The intensity of three compounds was increased in proportion to the apoptosis ratio and the correlation coefficients between apoptotic cell percentage and peak area was shown in Table 3. The ratio was apoptotic-dependent and consistent with the results from the flow cytometric analysis. On the other side, the intensity of decane has no relationship with apoptosis changed according to necrotic percentage increase, and could possibly be assigned to the necrotic phase (Fig. 2, Table 3). To date, the apoptosis/necrosis mechanisms for the identified 4 VOCs biomarkers have not been reported, and therefore further investigation of the function of VOCs in apoptosis/necrosis is required.

Additionally, the VOCs of RPMI-1640 and of RPMI-1640 supplemented with cisplatin were compared. However, no differential chromatogram was found between the two media (the data not shown), suggesting the changed VOCs were metabolites related to cytotoxic activity and not to cisplatin itself.

4. Conclusions

Cisplatin-induced cell death was successfully investigated by analyzing the VOCs of cisplatin-treated human lung carcinoma cell lines. The developed method differentiated apoptosis from necrosis, and the results were in excellent agreement with those of conventional flow cytometric measurement. The method eliminates the additional requirement of cell trypsination as well as the possibility of cell damage due to pipetting, centrifugation, and other like processes.

This paper provides evidence that VOCs can be used as sensitive biomarkers for determination of the mode of cell death. One of the advantages of using VOCs as biomarkers is that there is no need for any invasive and time-consuming procedure; this allows the cells to be reused for other subsequent experiments, obtains the reproducibility, and reduces costs. Monitoring the status of living cells in real time is another advantage; the change in cellular responses can be monitored over time from a single cell-culture sample since only the volatile compounds in the cell-culture atmosphere are utilized. In conclusion, the experiments described and exposited here have important implications with regard to the evaluation of other cellular metabolisms as well as to drug toxicity monitoring using VOCs as biomarkers.

Acknowledgement

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-331-E00313).

References

[1] A. Eastman, Cancer Cells 2 (1990) 275.
[2] T. Nomoto, K. Nishio, N. Saijo, Gan To Kagaku Ryoho 22 (1995 1719).
[3] A.H. Lau, Kidney Int. 56 (1999) 1295.
[4] D. Wang, S.J. Lippard, Nat. Rev. Drug Discov. 4 (2005) 307.
[5] M. Phillips, R.N. Cataneo, B.A. Ditkoff, P. Fisher, J. Greenberg, R. Gunawardena, C.S. Kwon, F. Rahbari-Oskoui, C. Wong, Breast J. 9 (2003) 184.
[6] L. Pauling, A.B. Robinson, R. Teranishi, P. Cary, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 2374.
[7] S.M. Gordon, J.P. Szidon, B.K. Krotoszynski, R.D. Gibbons, H.J. O'Neill, Clin. Chem. 31 (1985) 1278.
[8] H.J. O'Neill, S.M. Gordon, M.H. O'Neill, R.D. Gibbons, J.P. Szidon, Clin. Chem. 34 (1988) 1613.
[9] C. Deng, X. Zhang, N. Li, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 808 (2004) 269.
[10] R. Xue, L. Dong, S. Zhang, C. Deng, T. Liu, J. Wang, X. Shen, Rapid Commun. Mass Spectrom. 22 (2008) 1181.
[11] D. Zimmermann, M. Hartmann, M.P. Moyer, J. Nolte, J.I. Baumbach, Metabolomics 3 (2007) 13.
[12] L.C. Amorim, L.C.Z. de, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 853 (2007) 1.
[13] Validation of Analytical Procedures: Methodology Q2B, ICH Steering Committee, 6 November 1996, website http://www.ich.org/.
[14] V.M. Gonzalez, M.A. Fuertes, C. Alonso, J.M. Perez, Mol. Pharmacol. 59 (2001) 657.