Table 4. Interday Precision for the Determination of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA Adduct Levels in the Rat Liver DNA Samples on Five Different Days Using LC-MS/MS

	repeat analysis of ES-3′-8- dG adduct (nM)						
sample no.	day 1	day 2	day 3	day 4	day 5	mean (nmol/L)	RSD (%)
6	0.510	0.518	0.491	0.506	0.509	0.507 ± 0.010	1.96
7	0.431	0.443	0.450	0.432	0.459	0.443 ± 0.012	2.68
8	0.462	0.470	0.482	0.483	0.445	0.468 ± 0.016	3.39
9	0.474	0.490	0.480	0.458	0.476	0.476 ± 0.011	2.39
10	0.484	0.483	0.483	0.511	0.447	0.482 ± 0.023	4.74

	repeat analysis of ES-3′-N^2-dG adduct (nM)						
sample no.	day 1	day 2	day 3	day 4	day 5	mean (nmol/L)	RSD (%)
6	0.701	0.711	0.733	0.703	0.727	0.715 ± 0.014	1.97
7	0.522	0.597	0.597	0.586	0.546	0.569 ± 0.034	5.91
8	0.590	0.626	0.608	0.638	0.605	0.613 ± 0.019	3.06
9	0.711	0.706	0.705	0.678	0.661	0.692 ± 0.022	3.15
10	0.737	0.676	0.717	0.706	0.718	0.711 ± 0.022	3.13

	repeat analysis of ES-3′-N^6-dA adduct (nM)						
sample no.	day 1	day 2	day 3	day 4	day 5	mean (nmol/L)	RSD (%)
6	3.100	2.967	3.098	3.044	2.949	3.032 ± 0.071	2.36
7	2.286	2.333	2.236	2.339	2.250	2.289 ± 0.047	2.04
8	2.699	2.617	2.738	2.797	2.766	2.723 ± 0.069	2.55
9	2.444	2.455	2.546	2.506	2.526	2.495 ± 0.044	1.77
10	2.736	2.735	2.846	2.880	2.830	2.805 ± 0.066	2.36

IQL, respectively. The peak area ratio with respect to each surrogate standard was plotted, and the response was found to be linear (ES-3′-C8-dG, $y = 1.9707x − 0.0096$ and $y = 2.0318x − 0.0989$; ES-3′-N^2-dG, $y = 0.6359x + 0.0048$ and $y = 0.6336x − 0.0263$; and ES-3′-N^6-dA, $y = 1.8567x + 0.012$ and $y = 1.8205x + 0.065$) over the calibration range, from LOQ to 1.0 nM (low range) and from 0.1 to 10 nM (high range), with a correlation coefficient (r) of over 0.999. The average retention times of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA standards were 11.8 (RSD = 0.08%, n = 5), 12.7 (RSD = 0.12%, n = 5), and 15.1 min (RSD = 0.06%, n = 5), respectively. MRM chromatograms of the mixture of three adduct and their corresponding surrogate standards in 50% methanol at LOD and LOQ levels are shown in Figure 5.

As shown in Table 2, the average recoveries of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA from DNA sample in the livers of nontreated rats were 99.0, 100.1, and 101.7% for each adduct.

Analysis of ES-N^2-dG, C8-dG, and N^6-dA in the Livers DNA Extracted from Rats Treated with ES. The potential formation of ES adducts in livers of rats exposed to ES was assessed using the isotope dilution LC-ESI/MS/MS method. Typical selected ion recording (SIR) chromatograms are shown in Figure 6. No peaks indicative of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were observed in liver DNA extracted from control rats. In contrast, ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were detected at 11.8, 12.7, and 15.1 min in the liver DNA MRM chromatograms extracted from rats treated with 600 mg/kg ES for up to 4 weeks, respectively. Quantitative data are summarized in Table 3. The ES-3′-C8-dG, ES-3′-N^2-dG/10^6dG, and ES-3′-N^6-dA/10^6dA ratios were 3.5 ± 0.4, 4.8 ± 0.8, and 20.5 ± 1.6,

respectively. The interday sample variation of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA adduct levels analyzed on different days resulted in an average coefficient of variation (CV) of 3.0, 3.4, and 2.2% for different samples that were analyzed (Table 4). All of these adducts were not detected in the control group.

■ DISCUSSION

Phillips et al. have suggested the chemical structure of four ES-specific DNA adducts, ES-3′-N^2-dG, ES-3′-C8-dG, ES-1′-N^2-dG, and ES-3′-N^6-dA, using radio isotope-labeled nucleosides. The formation of these adducts in mouse liver treated with [^{14}C]-labeled ES was demonstrated by radioactive detection after HPLC fractionation.[8] Subsequently, LC-MS analysis by Punt et al. demonstrated the formation of three ES-dG adducts, ES-3′-N^2-dG, ES-3′-C8-dG, and ES-1′-N^2-dG, after in vitro nucleoside reaction.[7] However, the existence of the dA adduct reported previously and its chemical structure have not been confirmed using MS technique. In the present study, we examine dA adduct formation using LC-ESI/MS by taking advantage of reactive carbocation formation generated by acetylation of ES proposed by Punt et al.[7] In the reaction of dA with 1′-acetoxy-ES, two peaks including ions characteristic for the ES-dA adduct were observed by total ion chromatography. Subsequently, ^{1}H NMR analysis of large-scale synthesized and HPLC purified samples led to the conclusion that the major adduct was ES-3′-N^6-dA, in line with the previous report.[8] Although a minor and unknown ES-dA adduct was also found, its precise chemical structure could not be elucidated due to the low yield.

ES has been reported to have genotoxicity and carcinogenicity in the livers of mice of both sexes.[2] ES treatment at a dose of 600 mg/kg by gavage for 16 weeks induced significant developments of glutathione S-transferase placenta form (GST-P) foci in the liver of rats (unpublished data). To examine the precise quantity of ES-specific adducts in ES-hepatocarcinogenesis, we used interim samples (4 weeks) for the carcinogenicity study mentioned above. On the basis of previous reports, we attempted to detect ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA. Because appropriate sample preparations and a highly sensitive analytical method are necessary to detect modified DNA bases in genomic DNA, we developed a new analytical method by an isotope dilution LC-ESI/MS/MS method using SIR. ^{32}P-postlabeling analysis without internal standards for identifying the products neither provides structural characterization of adducts nor has sufficient selectivities. In addition, this assay requires radioactive γ-^{32}P-labeled ATP in the analytical process, which raises the necessity of rigorous handling. The LC-MS/MS technique using stable isotope adducts as internal standards achieves accurate quantification of DNA adducts along with structural characterization. The LOQs of ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA in our method were determined to be 2−5 adducts/10^8 unmodified dG or dA bases from a 150 mg liver sample. In addition, the high recoveries of these adducts in the wide range from LOQ level indicated that our new method enables precise adduct determination with the use of surrogate standards and is applicable to the detection of these compounds in animal tissue samples. As a result, our method was able to quantify these three adducts in liver DNA of rats treated with 600 mg/kg ES for 4 weeks, and we observed that the ES-3′-N^6-dA adduct is predominant. Phillips et al. have suggested that ES-3′-N^2-dG was a major adduct in the livers of mice treated with single dose of 1′-hydroxy-ES (12 μmol/mouse), a metabolite of ES. Considering that the present experiment was performed under carcinogenic conditions, it is highly probable that the status of DNA adduct formation observed in the present study may be reflected in the ES carcinogenesis. The modification at the N^6-position of dA has been reported following treatment with other potent mutagens including aristolochic acid,[22,23] polyaromatic hydrocarbon, benzo[c]phenanthrene,[29,30] and 5,6-dimathylchrysene.[31] It is likely that the formation of various types of chemical-specific base modifications is dependent on accessibility of the chemical (or the proximate form) to the reactive amino group between dG and dA in the DNA helical structure.[32] Those mutagens predominantly induce AT-TA transversion and AT-GC transition mutations.[25,33,34] Therefore, our unpublished data that the AT-GC transition mutation was predominant in ES-treated rat livers allow us to hypothesize that N^6-dA adducts might play a key role in ES mutagenicity. Thus, information regarding the precise concentrations of ES-specific DNA adducts discovered in the present study would be very helpful for further research on ES hepatocarcinogenesis.

Minor adducts of dG (ES-1′-N^2-dG) and dA (not identified) were hardly detected in in vivo samples even though such adducts are detectable in the reaction of 1′-acetoxy-ES with deoxynucleoside.[7] Dissociation of the ester group from an ester of 1-hydroxy-ES generates an electrophilic ion in which the positive charge may reside on the double bond between the 2-, 3- and 1-, 2-positions on the allyl chain. Nucleophilic attack by the purine bases of DNA at the 1′-position of ES would account for ES-1′-N^2-dG formation, while attack at the 3′-position would account for ES-3′-N^2-dG. Because dG modification at the 3′-position of

ES far from the benzene ring is predominant both in vivo and in vitro, it is conceivable that the priority of these reactions is determined by steric hindrance between DNA bases and ES. The suggestion that unknown minor dA adducts also might result from modification at the 1′-position of ES is in line with this hypothesis. Therefore, the fact that genomic DNA possesses more steric hindrance than nucleosides might explain the lack of detection of these minor adducts in vivo.

In conclusion, dA modified by ES was determined to be EG-3′-N^6-dA as the major adduct. ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA adducts can be identified and quantified by this new method, which may prove useful in other related studies.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-3-3700-9819. Fax: +81-3-3700-1425. E-mail: y-ishii@nihs.go.jp.

ABBREVIATIONS

ES, estragole; dG, deoxyguanosine; dA, deoxyadenosine; LC, liquid chromatography; ESI, electron spray ionization; MS, mass spectrometry; NMR, nuclear magnetic resonance; MS/MS, tandem mass spectrometry; SIR, selected ion recording; LOQ, limit of quantification; SULT, sulfotransferase; THF, tetrahydrofuran; DMSO, dimethylsulfoxide; DMF, dimethylformamide; TMS, trimethylsilane; MRM, multiple reaction monitoring; PDA, photodiode array; IDL, instrument detection limit; IQL, instrument quantification limit; LOD, limit of detection; GST-P, glutathione S-transferase placenta form.

REFERENCES

(1) Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., and Rogers, A. E. (2002) Safety assessment of allylalkoxybenzene derivatives used as flavouring substances-Methyl eugenol and estragole. *Food Chem. Toxicol.* 40, 851–870.

(2) Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pilot, H. C. (1976) Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1′-hydroxyestragole in the mouse and mutagenicity of 1′-aceotyestragole in bacteria. *J. Natl. Cancer Inst.* 57, 1323–1331.

(3) Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983) Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.* 43, 1124–1134.

(4) Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987) Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F₁ mice. *Cancer Res.* 47, 2275–2283.

(5) Jeurissen, S. M., Punt, A., Boersma, M. G., Bogaards, J. J. P., Fiamegos, Y. C., Schilter, B., Van Bladeren, P. J., Cnubben, N. H. P., and Rietjens, I. M. C. M. (2007) Human cytochrome P450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. *Chem. Res. Toxicol.* 20, 798–806.

(6) Fennell, T. R., Wiseman, R. W., Miller, J. A., and Miller, E. C. (1985) Major role of hepatic sulfotransferase activity in the metabolic activation, DNA adduct formation, and carcinogenicity of 1′-hydroxy-2′3′-dehydroestragole in infant male C57BL/6J x C3H/HeJ F1 mice. *Cancer Res.* 45, 5310–5320.

(7) Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. (2007) Tandem mass spectrometry analysis of N^2-(trans-isoestragol-3′-yl)-2′-deoxyguanosine as a strategy to study

species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. *Chem. Res. Toxicol. 20*, 991–998.

(8) Phillips, D. H., Reddy, M. V., Miller, E. C., and Adams, B. (1981) Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Res. 41*, 176–186.

(9) Phillips, D. H., Reddy, M. V., and Randerath, K. (1984) ^{32}P-postlabelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzens II. Newborn male B6C3F1 mice. *Carcinogenesis 5*, 1623–1628.

(10) Rnaderathe, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984) ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis 5*, 1613–1622.

(11) Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985) Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. *Cancer Res. 45*, 3096–3105.

(12) Prakash, A. S., Tran, H. P., Peng, C., Koyalamudi, S. R., and Dameron, C. T. (2000) Kinetics of DNA alkylation, depurination and hydrolysis of anti diol epoxide of benzo(a)pyrene and the effect of cadmium on DNA alkylation. *Chem.-Biol. Interact. 125*, 133–150.

(13) Moustacchi, E. (2000) DNA damage and repair: Consequences on dose-responses. *Mutat. Res. 464*, 35–40.

(14) Choi, J. Y., and Guengerich, F. P. (2005) Adduct size limits efficient and error-free bypass across bulky N2-guanine DNA lesions by human DNA polymerase eta. *J. Mol. Biol. 352*, 72–90.

(15) Wei, D., Maher, V. M., and McCormick, J. J. (1996) Site-specific excision repair of 1-nitrosopyrene-induced DNA adducts at the nucleotide level in the HPRT gene of human fibroblasts: Effect of adduct conformation on the pattern of site-specific repair. *Mol. Cell. Biol. 16*, 3714–3719.

(16) Singh, R., Gaskell, M., Le Pla, R. C., Kaur, B., Azim-Araghi, A., Roach, J., Koukouves, G., Souliotis, V. L., Kyrtopoulos, S. A., and Farmer, P. B. (2006) Detection and quantification of benzo[a]pyrene-derived DNA adducts in mouse liver by liquid chromatography-tandem mass spectrometry: comparison with 32P-postlabeling. *Chem. Res. Toxicol. 19*, 868–878.

(17) Pfau, W., Brockstedt, U., Söhren, K. D., and Marquardt, H. (1994) 32P-post-labelling analysis of DNA adducts formed by food-derived heterocyclic amines: Evidence for incomplete hydrolysis and a procedure for adduct pattern simplification. *Carcinogenesis 15*, 877–882.

(18) Lyle, T. A., Royer, R. E., Daub, G. H., and Vander Jagt, D. L. (1980) Reactivity-selectivity properties of reactions of carcinogenic electrophiles and nucleosides: Influence of pH on site selectivity. *Chem.-Biol. Interact. 29*, 197–207.

(19) Yuan, B., Cao, H., Jiang, Y., Hong, H., and Wang, Y. (2008) Efficient and accurate bypass of N2-(1-carboxyethyl)-2'-deoxyguanosine by DinB DNA polymerase in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A. 105*, 8679–8684.

(20) Xu, P., Oum, L., Geacintov, N. E., and Broyde, S. (2008) Nucleotide selectivity opposite a benzo[a]pyrene-derived N2-dG adduct in a Y-family DNA polymerase: A 5'-slippage mechanism. *Biochemistry 47*, 2701–2709.

(21) Stover, J. S., Chowdhury, G., Zang, H., Guengerich, F. P., and Rizzo, C. J. (2006) Translesion synthesis past the C8- and N2-deoxyguanosine adducts of the dietary mutagen 2-Amino-3-methylimidazo[4,5-f]quinoline in the NarI recognition sequence by prokaryotic DNA polymerases. *Chem. Res. Toxicol. 19*, 1506–1517.

(22) Chan, W., Yue, H., Poon, W. T., Chan, Y. W., Schmitz, O. J., Kwong, D. W., Wong, R. N., and Cai, Z. (2008) Quantification of aristolochic acid-derived DNA adducts in rat kidney and liver by using liquid chromatography-electrospray ionization mass spectrometry. *Mutat. Res. 646*, 17–24.

(23) Grollman, A. P., Shibutani, S., Moriya, M., Miller, F., Wu, L., Moll, U., Suzuki, N., Fernandes, A., Rosenquist, T., Medverec, Z.,

Jakovina, K., Brdar, B., Slade, N., Turesky, R. J., Goodenough, A. K., Rieger, R., Vukelić, M., and Jalaković, B. (2007) Aristolochic acid and the etiology of endemic (Balkan) nephropathy. *Proc. Natl. Acad. Sci. U.S.A. 104*, 12129–12134.

(24) Kim, S. J., Jajoo, H. K., Kim, H. Y., Zhou, L., Horton, P., Harris, C. M., and Harris, T. M. (1995) An efficient route to N^6 deoxyadenosine adducts of diol epoxides of carcinogenic polycyclic aromatic hydrocarbons. *Bioorg. Med. Chem. 3*, 811–822.

(25) Schmeiser, H. H., Scherf, H. R., and Wiessler, M. (1991) Activating mutations at codon 61 of the c-Ha-ras gene in thin-tissue sections of tumors induced by aristolochic acid in rats and mice. *Cancer Lett. 59*, 139–143.

(26) Schmeiser, H. H., Janssen, J. W., Lyons, J., Scherf, H. R., Pfau, W., Buchmann, A., Bartram, C. R., and Wiessler, M. (1990) Aristolochic acid activates ras genes in rat tumors at deoxyadenosine residues. *Cancer Res. 50*, S464–S469.

(27) Nakae, D., Mizumoto, E., Kobayashi, E., Noguchi, O., and Konishi, Y. (1995) Improved genomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. *Cancer Lett. 97*, 233–239.

(28) Ishii, Y., Okamura, T., Inoue, T., Fukuhara, K., Umemura, T., and Nishikawa, A. (2010) Chemical structure determination of DNA bases modified by active metabolites of lucidin-3-O-primeveroside. *Chem. Res. Toxicol. 23*, 134–141.

(29) Yakovleva, L., Handy, C. J., Sayer, J. M., Pirrung, M., Jerina, D. M., and Shuman, S. (2004) Benzo[c]phenanthrene adducts and nogalamycin inhibit DNA transesterification by vaccinia topoisomerase. *J. Biol. Chem. 279*, 23335–23342.

(30) Ponten, I., Sayer, J. M., Pilcher, A. S., Yagi, H., Kumar, S., Jerina, D. M., and Dipple, A. (1999) Sequence context effects on mutational properties of cis-opened benzo[c]phenanthrene diol epoxide-deoxyadenosine adducts in site-specific mutation studies. *Biochemistry 38*, 1144–1152.

(31) Misra, B., Amin, S., and Hecht, S. S. (1992) Dimethylchrysene diol epoxides: Mutagenicity in Salmonella typhimurium, tumorigenicity in newborn mice, and reactivity with deoxyadenosine in DNA. *Chem. Res. Toxicol. 5*, 248–254.

(32) Blake, R. D. (2005) *Informational Biopolymers of Genes and Gene Expression*, p 290, University Science Books, CA.

(33) Page, J. E., Szeliga, J., Amin, S., Hecht, S. S., and Dipple, A. (1995) Mutational spectra for 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxides in the supF gene of pSP189. *Chem. Res. Toxicol. 8*, 143–137.

(34) Schmeiser, H. H., Janssen, J. W., Lyons, J., Scherf, H. R., Pfau, W., Buchmann, A., Bartram, C. R., and Wiessler, M. (1990) Aristolochic acid activates ras genes in rat tumors at deoxyadenosine residues. *Cancer Res. 50*, S464–S469.