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Profiling of *cis*-Diol-containing Nucleosides and Ribosylated Metabolites by Boronate-affinity Organic-silica Hybrid Monolithic Capillary Liquid Chromatography/Mass Spectrometry

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RNA contains a large number of modified nucleosides. In the metabolic re-exchange of RNA, modified nucleosides cannot be recycled and are thus excreted from cells into biological fluids. Determination of endogenous modified nucleosides in biological fluids may serve as non-invasive cancers diagnostic methods. Here we prepared boronate-affinity organic-silica hybrid capillary monolithic column (BOHCMC) that exhibited excellent selectivity toward the *cis*-diol-containing compounds. We then used the prepared BOHCMC as the on-line solid-phase microextraction (SPME) column and developed an on-line SPME-LC-MS/MS method to comprehensively profile *cis*-diol-containing nucleosides and ribosylated metabolites in human urine. Forty-five *cis*-diol-containing nucleosides and ribosylated metabolites were successfully identified in human urine. And five ribose conjugates, for the first time, were identified existence in human urine in the current study. Furthermore, the relative quantification suggested 4 *cis*-diol-containing compounds (5′-deoxy-5′-methylthioadensine, N^4-acetylcytidine, 1-ribosyl-*N*-propionylhistamine and N^2,N^2,7-trimethylguanosine) increased more than 1.5 folds in all the 3 types of examined cancers (lung cancer, colorectal cancer, and nasopharyngeal cancer) compared to healthy controls. The on-line SPME-LC-MS/MS method demonstrates a promising method for the comprehensive profiling of *cis*-diol-containing ribose conjugates in human urines, which provides an efficient strategy for the identification and discovery of biomarkers and may be used for the screening of cancers.

RNA contains a large number of modified nucleosides that are formed post-transcriptionally by various modification enzymes[1]. In the metabolic re-exchange of RNA, hydrolytic enzymes such as ribonucleases and phosphatases release normal and modified nucleosides during RNA regeneration. And normal nucleosides undergo reuse and degradation; whereas, modified nucleosides cannot be recycled as normal nucleosides for RNA synthesis and are thus excreted from cells into biological fluids[2]. The abnormal level of modified nucleosides has been reported to be associated with carcinogenesis[3], dyskeratosis congenital[4], diabetes[5,6] and Alzheimer's disease[7]. In this respect, determination of endogenous modified nucleosides in biological fluids have attracted considerable interest in recent years owing to their usefulness as non-invasive diagnostic and/or follow-up methods for certain pathologies.

So far, some analytical methods have been developed for the analysis of nucleosides and their derivatives in biological fluids, such as high-performance liquid chromatography (HPLC) with UV[8], radioactivity[9], or mass spectrometry (MS) detection[10,11], and capillary electrophoresis (CE) with UV[12] or MS detection[13]. UV absorbance-based detection makes the identification of compounds difficult. HPLC with radioactivity detection is sensitive, but involves in the use of radioactive materials. Mass spectrometry provides structural information and has been used for analyses of purine and pyrimidine nucleoside antiviral agents and naturally occurring nucleo-

sides[14]. However, due to the low abundance of modified nucleosides present in biological fluids as well as the serious matrix interferences of biological samples, comprehensive profiling of modified nucleosides in biological fluids is still challenging in clinical research.

Boronic acids are important ligands for the isolation and sensing of *cis*-diol-containing biomolecules, such as saccharides[15], glycoproteins[16], and nucleosides[17]. In this respect, boronic acids are widely used in the construction of functional materials, including nanoparticles[18], nanotubes[19], polymer brushes[20], and monoliths[21]. The primary mechanism of the affinity capturing of *cis*-diol-containing compounds by boronic acids mainly relies on the covalent binding of *cis*-diol moieties with boronic acids to form five- or six membered cyclic esters in an alkaline aqueous solution; while the esters dissociate when surrounding pH is changed to acidic. Duo to the specific capturing towards *cis*-diol-containing biomolecules and relative easy desorption of target compounds, affinity chromatography with boronic acids ligands could be a promising strategy for the selective enrichment of low abundant modified nucleosides present in biological fluids.

Herein, using the sol-gel combined with "thiol-ene" click reaction, we prepared boronate-affinity organic-silica hybrid capillary monolithic column (BOHCMC) that exhibited excellent selectivity toward the *cis*-diol-containing compounds. We then used the prepared BOHCMC as the on-line solid-phase microextraction (SPME) column and developed an on-line SPME-LC-MS/MS method to profile *cis*-diol-containing nucleosides and ribosylated metabolites in human urine (Figure 1). And 45 *cis*-diol-containing nucleosides and ribosylated metabolites were successfully identified. Furthermore, we performed the relative quantification of these identified *cis*-diol-containing nucleosides and ribosylated metabolites in urines of healthy controls and other 3 types of cancer patients, including lung cancer, colorectal cancer and nasopharyngeal cancer (Table S1 in Supporting Information).

Results

Characterization of BOHCMC. Recently, our group successfully developed a novel method to prepare organic-silica hybrid capillary monolithic column by sol-gel combined with "thiol-ene" click reaction, which allows higher yield and less by-products compared to other methods and can be performed under mild conditions[22]. Using the "thiol-ene" click reaction, here we successfully prepared BOHCMC in one-pot synthesis with Tetramethoxysilane (TMOS) and 3-mercaptopropyltrimethoxysilane (MPTMS) as functional silane coupling reagents (Figure 2A). The preparation strategy also notably simplified the construction of the monolith.

The morphology of the prepared BOHCMC was firstly examined by scanning electron microscopy (SEM). As shown in Figure S1A in Supporting Information, the formed monolith was homogeneous and attached well to the inner wall of the capillary, which can provide effective mass transfer and high stability. The specific surface area of the capillary monolithic column was 247 m^2/g with 4.9 nm mesoporous distribution examined by nitrogen adsorption-desorption experiments (Figure S1B in Supporting Information).

Performance of the BOHCMC toward *cis*-diol-containing compounds. The selectivity, extraction efficiency, extraction capacity and stability of the BOHCMC were investigated.

The selectivity of the BOHCMC was evaluated using 2′-deoxyadenosine (dA) and adenosine (rA) as the analytes. The results showed that rA retained well on the BOHCMC since the *cis*-diol of rA can readily react with 3-acrylamidophenylboronic acid on BOGCMC to form cyclic boronate esters at pH 8; while dA had no retention and was eluted out directly (Figure S1C in Supporting Information). After switching pH to acidity (0.1% formic acid in water), the structure of cyclic boronate esters was destroyed and rA was fast eluted out (Figure S1C in Supporting Information). Therefore, the prepared BOHCMC exhibited good selectivity toward the *cis*-diol-containing compound of rA; while the compound that lacks of *cis*-diol structure (dA) cannot retain.

The extraction capacity of the BOHCMC was evaluated by stepwise increase of the sample volume of rA (100 μg/mL). A curve was made by plotting the peak area of rA versus the injection volume (Figure S2 in Supporting Information). The results showed that the peak area proportionally increased as the injection volume increased from 0.1 to 2 μL. While further increase of the injection volume of rA did not result in the increase of the peak area of rA. Therefore, the extraction capacity was estimated to be 500 μg/g based on the adsorbed amount of rA and the weight of the monolith.

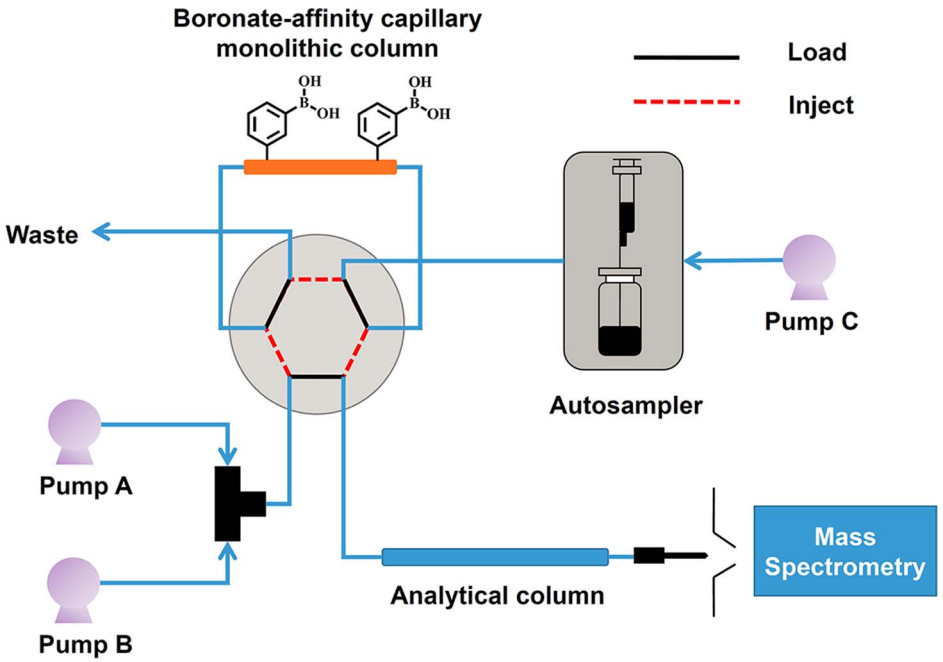

Figure 1 | Scheme of the on-line SPME-LC-MS/MS system.

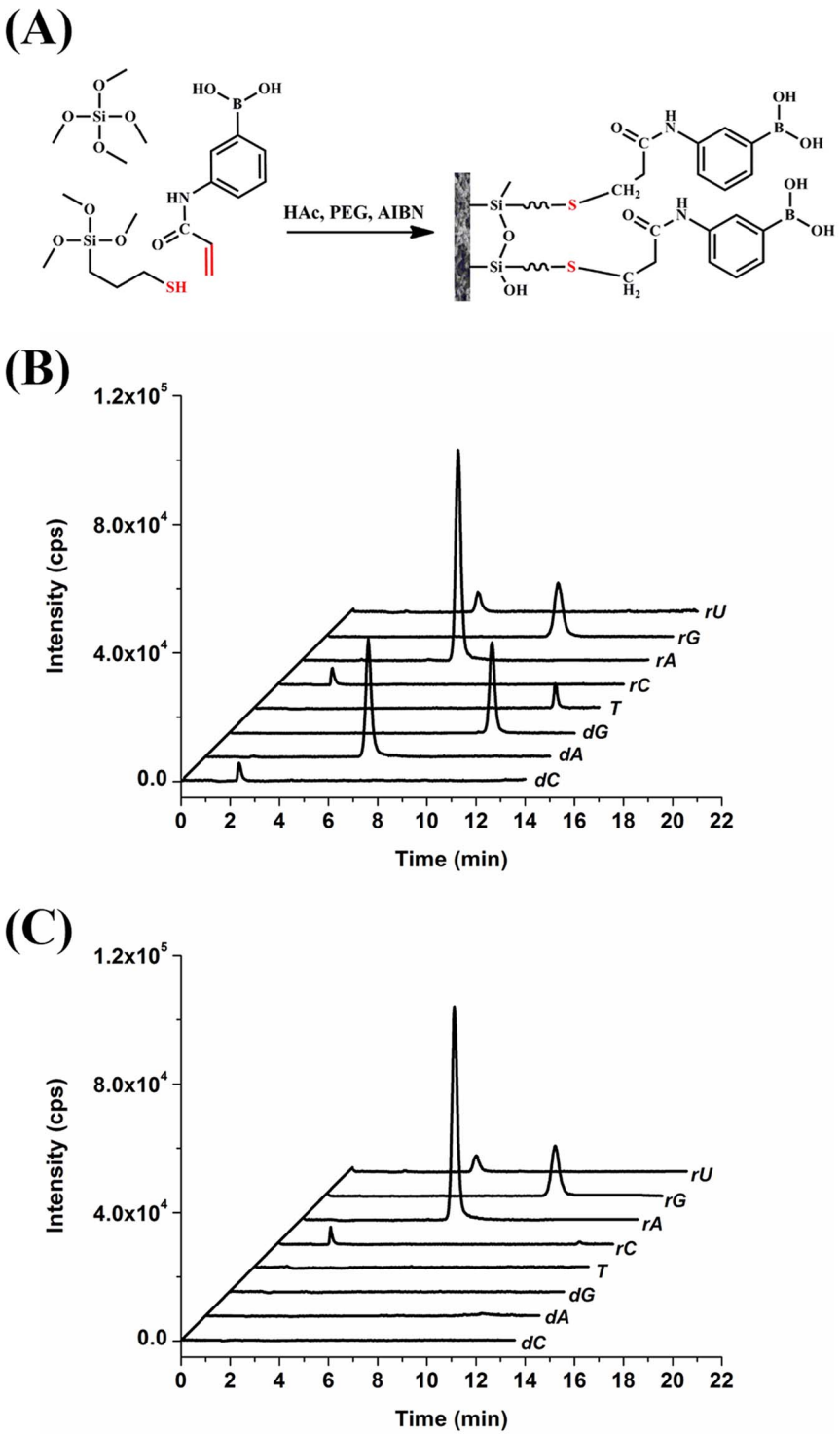

Figure 2 | Preparation and characterizations of the BOHCMC. (A) Schematic procedure for the preparation of BOHCMC by sol-gel method combined with "thiol-ene" click reaction. (B) Analysis of eight normal nucleosides by LC-MS/MS. (C) Analysis of eight normal nucleosides by on-line-SPME-LC-MS/MS under optimized conditions. BOHCMC: 10 cm-long, 250 μm i.d. × 360 μm o.d. Sample concentration, 0.05 μg/mL for each nucleoside.

The BOHCMC was used three times per week for consecutive four weeks to examine the stability, which was evaluated by the relative standard deviations (RSDs) of retention times and the peak areas of rA. The results showed that the RSDs of the retention time and peak areas of rA was 2.4% and 4.3%, respectively, demonstrating BOHCMC maintained good stability (Figure S3 in Supporting Information).

Optimization of on-line SPME conditions. The detailed experiments for the optimization of on-line SPME conditions can be found in Supporting Information. Under optimized conditions (loading flow rate, 5 μL/min; washing volume, 35 μL; desorption volume, 25 μL. Figure S4 in Supporting Information), we evaluated the extraction efficiency of BOHCMC using dA, T, dC, dG, rA, rU, rC and rG as analytes. As shown in Figure 2B and 2C, all the *cis*-diol-containing rA, rU, rC and rG can be well recovered after enrichment, while dA, T, dC and dG that don't contain *cis*-diol were efficiently removed. The calculated recoveries of 14 ribose conjugate standards at three different concentrations (25 ng/mL, 100 ng/mL and 500 ng/mL)

Table 1 | Identified 45 urinary ribose conjugates by on-line SPME-LC-MS/MS method or LC-MS/MS method

No.	Formula	Name	Product ions		Retention (min)	Without on-line SPME[a]
1	$C_{10}H_{14}N_2O_6$	5-Methyluridine	259.0940	127.0495	5.6	
2	$C_{10}H_{14}N_2O_6$	6-Methyluridine	259.0935	127.0493	6.6	
3	$C_{10}H_{13}N_5O_4$	Adenosine	268.1033	136.0612	7.4	
4	$C_{11}H_{15}N_5O_5$	1-Methylguanosine	298.1153	166.0710	12.2	
5	$C_{10}H_{13}N_5O_5$	Guanosine	284.0979	152.0647	12.3	
6	$C_{11}H_{14}N_4O_5$	1-Methylinosine	283.1031	151.0608	12.3	
7	$C_{11}H_{15}N_5O_4$	1-Methyladenosine	282.1187	150.0768	12.4	
8	$C_{11}H_{15}N_5O_5$	N^2-methylguanosine	298.1131	166.0717	12.6	
9	$C_{12}H_{17}N_5O_5$	N^2,N^2-dimethylguanosine	312.1289	180.0874	13.5	
10	$C_{10}H_{14}N_2O_6$	3-Methyluridine	259.0931	127.0499	13.5	
11	$C_{11}H_{15}N_5O_3S$	5′-Deoxy-5′-methylthioadensine	298.0955	136.0608	13.9	
12	$C_{14}H_{17}N_5O_8$	Succinyladenosine	384.1150	252.0732	15.4	
13	$C_{11}H_{15}N_5O_4$	N^6-methyladenosine	282.1185	150.0761	17.2	
14	$C_{10}H_{12}N_2O_8$	Orotidine	289.0664	157.0231	2.4	Not found
15	$C_{10}H_{12}N_4O_7$	Urate-3-ribonucleoside	301.0751	169.0332	2.4	Not found
16	$C_{11}H_{15}N_2O_5^+$	Nicotinamide riboside	255.0962	123.0546	2.5	Not found
17	$C_{11}H_{17}N_3O_6$	n-Ribosylhistidine	288.1198	156.0766	2.5	Not found
18	$C_9H_{13}N_3O_5$	Cytidine	244.0918	112.0499	3.0	Not found
19	$C_9H_{12}N_2O_6$	Pseudouridine	245.0761	113.0336	4.8	Not found
20	$C_9H_{12}N_2O_6$	Uridine	245.0786	113.0356	5.0	Not found
21	$C_{11}H_{15}N_3O_7$	5-Carbamoylmethyluridine	302.1202	170.0845	6.5	Not found
22	$C_{10}H_{14}N_4O_5$	6-Hydroxyl-1,6-dihydropurine ribonucleoside	271.0995	139.0589	7.8	Not found
23	$C_{13}H_{19}N_5O_5$	1-Methyl-N^2-ethylguanosine	326.1439	194.1046	8.4	Not found
24	$C_9H_{14}N_2O_6$	5,6-Dihydrouridine	247.0907	115.0499	8.5	Not found
25	$C_{10}H_{15}N_3O_5$	5-Methylcytidine	258.1074	126.0649	8.6	Not found
26	$C_{10}H_{12}N_4O_5$	Inosine	269.0880	137.0443	10.0	Not found
27	$C_{11}H_{12}N_2O_6$	Ribosylpyridinonecarboxamide	271.0913	139.0491	10.9	Not found
28	$C_{10}H_{12}N_4O_6$	Xanthosine	285.0836	153.0432	12.1	Not found
29	$C_{10}H_{13}N_5O_6$	8-Oxo-Guanosine	300.0919	168.0499	12.9	Not found
30	$C_{10}H_{15}N_3O_5$	3-Methylcytidine	258.1066	126.0647	13.1	Not found
31	$C_{11}H_{17}N_3O_6$	5-Methylaminomethyluridine	288.1181	126.0745	13.1	Not found
32	$C_{10}H_{13}N_5O_3$	5′-Deoxyadenosine	252.1075	136.0628	13.2	Not found
33	$C_{15}H_{20}O_7$	3-Hydroxychavicol 1-glucoside	313.1297	151.0744	13.5	Not found
34	$C_{11}H_{17}N_3O_5S$	5-Methylaminomethyl-2-thiouridine	304.0968	172.0513	14.6	Not found
35	$C_{12}H_{16}N_2O_9$	Uracil-5-oxyacetic acid methylester	333.0911	201.0495	14.9	Not found
36	$C_{11}H_{15}N_3O_6$	N^4-acetylcytidine	286.1015	154.0601	15.1	Not found
37	$C_{12}H_{19}N_3O_5$	1-Ribosyl-*N*-acetylhistamine	286.1377	154.0983	17.0	Not found
38	$C_{15}H_{20}N_6O_8$	N^6-threonylcarbamoyladenosine	413.1407	281.1013	17.1	Not found
39	$C_{12}H_{17}N_3O_5S$	N^6-Methyl-2-methylthioadenosine	312.1106	164.0917	17.6	Not found
40	$C_{11}H_{17}N_5O_5$	7-Methylguanosine	300.1275	168.0646	21.0	Not found
41	$C_{16}H_{20}N_2O_6$	4-[(1H-imidazol-2-yl)methyl]phenol-1-glucoside	337.1382	175.0846	22.6	Not found
42	$C_{15}H_{21}N_5O_4$	N^6-isopentenyladenosine	336.1646	204.1262	22.9	Not found
43	$C_{13}H_{20}N_5O_5^+$	N2,N2,7-trimethylguanosine	326.1451	194.1001	23.7	Not found
44	$C_{12}H_{19}N_5O_5$	N^2,7-dimethylguanosine	314.1441	182.1021	29.6	Not found
45	$C_{13}H_{21}N_3O_5$	1-Ribosyl-*N*-propionylhistamine	300.1565	168.1107	30.0	Not found

a, the same urine sample was analyzed by LC-MS/MS without on-line SPME.

were between 81.3% and 99.7% (Table S2 in Supporting Information), demonstrating good extraction efficiency of BOHCMC towards *cis*-diol-containing compounds were achieved.

Method validation. The reproducibility of the method was evaluated by the measurement of intra- and inter-day precisions. The intra- and inter-day RSDs were calculated by measuring 14 standards at the concentration of 0.2 mg/mL by on-line SPME-LC-MS/MS method. Three parallel analysis over a day gave the intra-day RSDs, and the inter-day RSDs were determined on 3 consecutive days. The results showed that both intra- and inter-day RSDs were less than 12.4% (Table S3 in Supporting Information), demonstrating good reproducibility were achieved.

The reproducibility of BOHCMC was evaluated by the measurement of batch-to-batch precisions. The RSDs of retention times and peak area ratios of 14 standards using 3 different batches of BOHCMC were calculated. The results showed that RSDs of retention times and peak area ratios were less than 3.2% and 13.1% respectively (Table S4 in Supporting Information), demonstrating good reproducibility of BOHCMC.

Determination of ribose conjugates in urine. We first profiled the *cis*-diol-containing nucleosides and ribosylated metabolites in urine by the developed on-line SPME-LC-MS/MS method. In this respect, a pooled sample that included urines from 10 lung cancer patients, 10 colorectal cancer patients, 10 nasopharyngeal cancer patients and 10 healthy controls was used. The results showed that 45 *cis*-diol-containing nucleosides and ribosylated metabolites were identified by tandem mass spectrometry analysis (Table 1). Among the 45 identified compounds, 14 *cis*-diol-containing ribose conjugates were further confirmed using the commercial standards through comparing the retention times and fragmentation ions (Figure S5 in Supporting Information). The fragmentation ions of the other 31 *cis*-diol-containing ribose conjugates were shown in Figure S6 in Supporting Information.

By comparison, we also profiled the *cis*-diol-containing ribose conjugates in urine without using on-line SPME. The results showed that the quantities of the peaks obtained using on-line SPME are much more than that without on-line SPME (Figure S7 in Supporting Information). In addition, only 13 *cis*-diol-containing

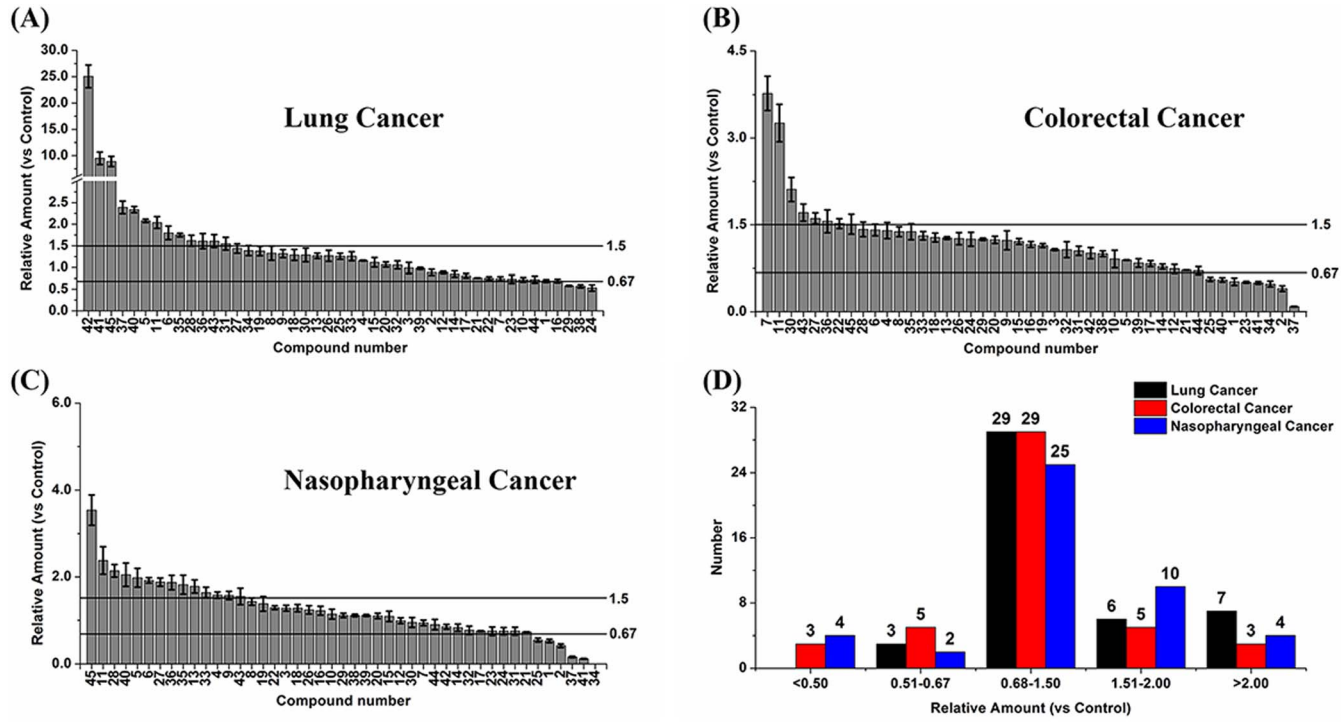

Figure 3 | Relative amounts ratio of *cis*-diol-containing nucleosides and ribosylated metabolites in (A) lung cancer, (B) colorectal cancer and (C) nasopharyngeal cancer compared to heathy controls. (D) Bar graphs for the distribution of the relative amounts ratio of *cis*-diol-containing nucleosides and ribosylated metabolites.

ribose conjugates were identified by direct analysis without on-line SPME enrichment (Table 1), which may be attributed to the serious matrix interference of urine as well as the low abundance of the modified nucleosides present in urine.

Contents change of ribose conjugates in urine between healthy controls and cancer patients. Using the developed method, we further compared the amounts of the 45 *cis*-diol-containing nucleosides and ribosylated metabolites in urine between healthy controls and 3 types of cancers, including lung cancer, colorectal cancer and nasopharyngeal cancer. In this respect, pooled samples of healthy controls (n=10) or each type of cancer (n=10 for each type of cancer) were prepared to minimize the variation between individuals. Normally 5 to 15 individual samples are used to form one pooled sample[23–25], therefore here we put 10 individual samples to form a pooled sample. In addition, to avoid the effects of drugs treatment on the alterations of ribose conjugates, all the cancer patients were diagnosed for the first time and hadn't been given any treatments. Before analysis, we quantified creatinine in each pooled sample according to previously described method[26] (Table S5 in Supporting Information). The nucleosides and ribosylated metabolites are related to the urinary creatinine concentration, which is a standard manner to normalize urinary metabolites since the excretion of creatinine is rather constant over a longer time interval. To examine the contents change of ribose conjugates between healthy controls and cancer patients, DHzR was used as an internal standard to compensate the instrumental variation. Then the relative content ratio of each identified *cis*-diol-containing compound between each type of cancer and healthy controls can be obtained (Figure 3 and Table 2).

To further validate the results obtained by the developed on-line SPME-LC-MS/MS method, we also examined the contents change of 13 ribose conjugates between healthy controls and cancer patients by direct LC-MS/MS (AB 3200 QTRAP mass spectrometer) analysis without on-line SPME under multiple reaction monitoring (MRM) mode (detailed experimental procedure can be found in Supporting Information). The results showed that the relative errors (REs) between these two different methods were under 13.3% (Table S6 in Supporting Information), demonstrating the contents change of the ribose conjugates measured by the two different methods are comparable.

The results showed that compared to healthy controls, 13, 8 and 14 identified ribose conjugates increased more than 1.5 folds in the urine of lung cancer, colorectal cancer and nasopharyngeal cancer, respectively. On the contrary, 3, 8 and 6 identified ribose conjugates decreased more than 1.5 folds in the urine of lung cancer, colorectal cancer and nasopharyngeal cancer, respectively.

Next we evaluated the relative contents change of urinary ribose conjugates between different types of cancer and healthy controls. In our current study, we found N^4-acetylcytidine increased in urines of all the examined types of cancer, which was consistent with previous reports that the level of N^4-acetylcytidine dramatically increased in multiple cancers, including lung, breast, kidney, colon, and colorectal cancers[27,28]. It was worth noting that here we also identified, for the first time, another three ribose conjugates of 5′-deoxy-5′-methylthioadenosine, 1-ribosyl-*N*-propionylhistamine and N^2,N^2,7-trimethylguanosine that were increased in urines of all the tested types of cancers (Table 2 and Figure 4).

As in lung cancer, we identified 13 increased ribose conjugates, among which 1-methylinosine and N^4-acetylcytidine were previously reported to be increased in urine of lung cancer patients[27]. And in colorectal cancer, we identified 8 increased ribose conjugates, among which 1-methyladenosine and N^4-acetylcytidine were previously reported to be increased in urine of colorectal cancer patients[29,30]. While so far there is no study reporting the identification of modified nucleosides in urine of nasopharyngeal cancer; and our study presented the first report for the identification of 14 increased and 6 decreased ribose conjugates, respectively, in urine of nasopharyngeal cancer.

Discussion

RNA molecules contain various modified nucleosides in addition to normal nucleosides. During RNA turnover, free nucleosides are gen-

Table 2 | The relative contents of 45 identified *cis*-diol-containing compounds in urines of 3 types of cancer patients over healthy controls

	Identified *cis*-diol-containing compounds	Content ratios (*vs* Healthy controls)		
		Lung cancer	Colorectal cancer	Nasopharyngeal cancer
1	5-Methyluridine	0.69 ± 0.03	0.52 ± 0.06	0.53 ± 0.04
2	6-Methyluridine	0.81 ± 0.07	0.40 ± 0.05	0.42 ± 0.04
3	Adenosine	0.99 ± 0.13	1.07 ± 0.02	1.28 ± 0.07
4	1-Methylguanosine	1.16 ± 0.01	1.40 ± 0.14	1.58 ± 0.07
5	Guanosine	2.08 ± 0.04	0.89 ± 0.00	1.98 ± 0.22
6	1-Methylinosine	1.80 ± 0.16	1.41 ± 0.10	1.92 ± 0.06
7	1-Methyladenosine	0.74 ± 0.05	3.77 ± 0.29	0.94 ± 0.07
8	N^2-methylguanosine	1.33 ± 0.16	1.38 ± 0.09	1.43 ± 0.09
9	N^2, N^2-dimethylguanosine	1.32 ± 0.09	1.23 ± 0.16	1.57 ± 0.10
10	3-Methyluridine	0.72 ± 0.05	0.91 ± 0.15	1.14 ± 0.11
11	5′-Deoxy-5′-methylthioadensine	2.04 ± 0.14	3.26 ± 0.32	2.38 ± 0.32
12	Succinyladenosine	0.89 ± 0.03	0.74 ± 0.08	0.99 ± 0.07
13	N^6-methyladenosine	1.27 ± 0.06	1.27 ± 0.02	1.78 ± 0.15
14	Orotidine	0.85 ± 0.07	0.78 ± 0.04	0.83 ± 0.08
15	Urate-3-ribonucleoside	1.12 ± 0.11	1.21 ± 0.05	1.09 ± 0.12
16	Nicotinamide riboside	0.69 ± 0.04	1.16 ± 0.05	1.22 ± 0.10
17	n-Ribosylhistidine	0.81 ± 0.05	0.83 ± 0.05	0.75 ± 0.01
18	Cytidine	1.29 ± 0.13	1.28 ± 0.08	1.28 ± 0.09
19	Pseudouridine	1.37 ± 0.11	1.13 ± 0.04	1.37 ± 0.17
20	Uridine	1.06 ± 0.06	1.24 ± 0.06	1.10 ± 0.06
21	5-Carbamoylmethyluridine	0.76 ± 0.03	0.72 ± 0.02	0.73 ± 0.06
22	6-Hydroxyl-1,6-dihydropurine ribonucleoside	0.75 ± 0.04	1.52 ± 0.09	1.29 ± 0.05
23	1-Methyl-N^2-ethylguanosine	0.73 ± 0.10	0.51 ± 0.02	0.75 ± 0.10
24	5,6-Dihydrouridine	0.53 ± 0.07	1.25 ± 0.12	0.75 ± 0.08
25	5-Methylcytidine	1.26 ± 0.07	0.56 ± 0.04	0.55 ± 0.05
26	Inosine	1.27 ± 0.13	1.26 ± 0.11	1.24 ± 0.01
27	Ribosylpyridinonecarboxamide	1.44 ± 0.11	1.61 ± 0.09	1.88 ± 0.10
28	Xanthosine	1.62 ± 0.13	1.42 ± 0.13	2.14 ± 0.14
29	8-Oxo-Guanosine	0.58 ± 0.01	1.25 ± 0.02	1.11 ± 0.05
30	3-Methylcytidine	1.29 ± 0.16	2.11 ± 0.21	0.95 ± 0.11
31	5-Methylaminomethyluridine	1.55 ± 0.15	1.05 ± 0.08	0.75 ± 0.09
32	5′-Deoxyadenosine	1.06 ± 0.09	1.07 ± 0.14	0.77 ± 0.10
33	3-Hydroxychavicol 1-glucoside	1.26 ± 0.10	1.31 ± 0.07	1.64 ± 0.12
34	5-Methylaminomethyl-2-thiouridine	1.39 ± 0.11	0.48 ± 0.05	0.005 ± 0.00
35	Uracil-5-oxyacetic acid methylester	1.75 ± 0.04	1.38 ± 0.14	1.82 ± 0.22
36	N^4-acetylcytidine	1.61 ± 0.17	1.56 ± 0.20	1.87 ± 0.17
37	1-Ribosyl-*N*-acetylhistamine	2.39 ± 0.15	0.09 ± 0.01	0.16 ± 0.02
38	N^6-threonylcarbamoyladenosine	0.57 ± 0.04	1.00 ± 0.05	1.11 ± 0.03
39	N^6-Methyl-2-methylthioadenosine	0.98 ± 0.03	0.84 ± 0.07	1.11 ± 0.02
40	7-Methylguanosine	2.34 ± 0.07	0.55 ± 0.04	2.05 ± 0.27
41	4-((1H-imidazol-2-yl)methyl)phenol-1-glucoside	9.49 ± 1.19	0.50 ± 0.02	0.12 ± 0.01
42	N^6-isopentenyladenosine	25.04 ± 2.18	1.01 ± 0.01	0.85 ± 0.06
43	N2,N2,7-trimethylguanosine	1.61 ± 0.15	1.71 ± 0.15	1.55 ± 0.19
44	N^2,7-dimethylguanosine	0.72 ± 0.08	0.71 ± 0.07	0.90 ± 0.12
45	1-Ribosyl-*N*-propionylhistamine	8.90 ± 0.97	1.51 ± 0.17	3.54 ± 0.35

erated by the hydrolytic action of ribonucleases and phosphatases. Normal nucleosides can be re-utilized to form nucleotide triphosphates which are incorporated into nucleic acids, or further degraded to form uric acid and β-alanine[31]. Modified nucleosides, in contrast, cannot be reused in *de novo* RNA synthesis or further metabolized[32]; therefore, modified nucleosides circulate in blood stream and are then excreted into urine. Consequently, the levels of the modified nucleosides reflect RNA turnover in organism[33,34]. It has been postulated that diseases may influence the rate of RNA turnover and thus be seen in the levels of excreted modified nucleosides[35]. Based on these biochemical findings, modified nucleosides have been proposed and evaluated as tumor biomarkers.

Here we developed a on-line SPME-LC-MS/MS method for the comprehensive profiling of *cis*-diol-containing nucleosides and ribosylated metabolites in urine. The BOHCMC exhibited excellent performance on the selective capturing of *cis*-diol-containing compounds. The unique property of the boronate-affinity on-line SPME notably improved the detection of the *cis*-diol-containing compounds by enriching target analytes as well as removing matrix inteference during LC-MS/MS analysis. Using the developed on-line SPME-LC-MS/MS method, 45 *cis*-diol-containing compounds were succesfully enriched and identified in a single LC-MS/MS analysis, which is much better than previous reports[8,10,27,28,30,36,37].

In these identified *cis*-diol-containing compounds, 5 modified nucleosides and ribosylated metabolites were first discorved in human urine, including 3-hydroxychavicol 1-glucoside, 5-carbamoylmethyluridine, 6-hydroxyl-1,6-dihydropurine ribonucleoside, 1-ribosyl-*N*-acetylhistamine and 4-((1H-imidazol-2-yl)methyl)phenol-1-glucoside, which extends the divisity of the modified nucleosides and ribosylated metabolites present in human urine. It is worth noting that many ribose conjuates were also found decrease in urine of cancer patients, which may reflect the abnormal metabolism of nucleic acids. However, further exploration is needed to elucidate the mechanism.

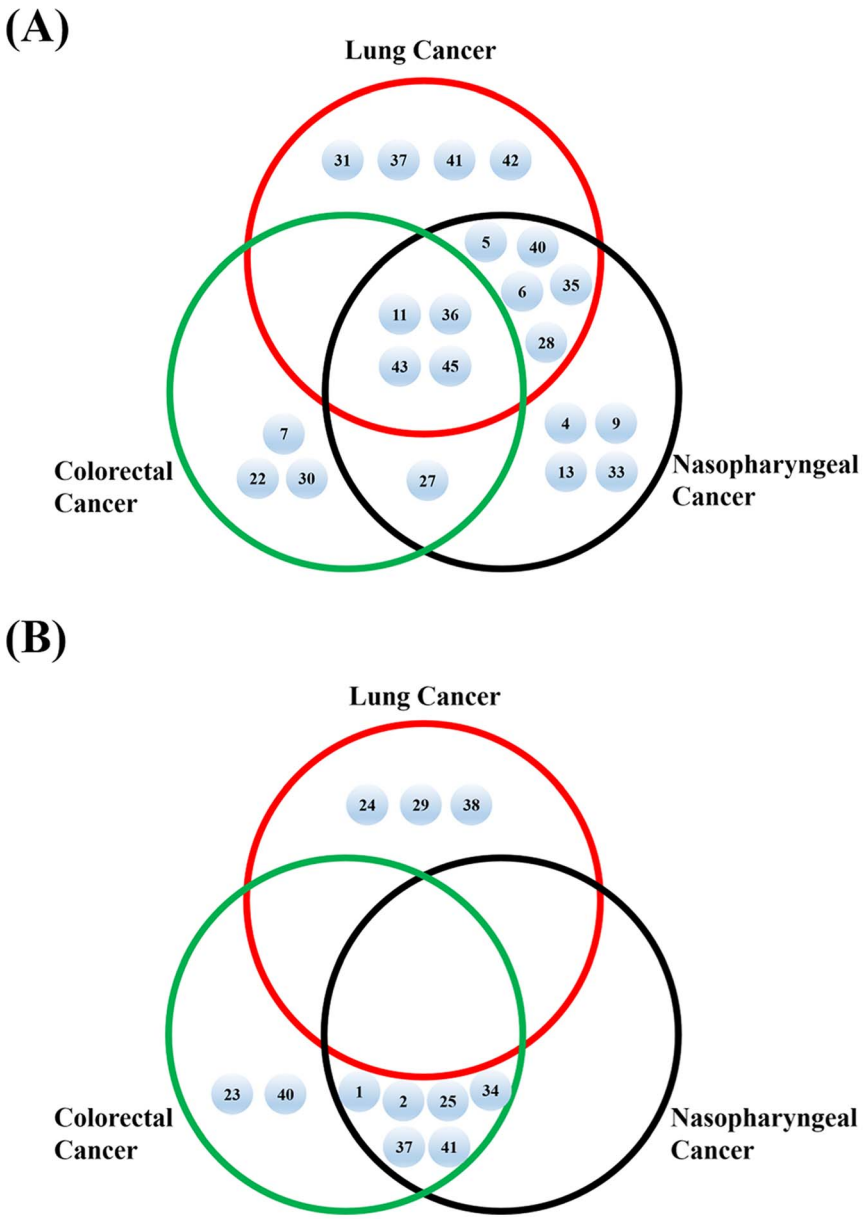

Figure 4 | The ribose conjugates that increased or decreased more than 1.5 folds in different cancer types compared to healthy controls. (A) The identified ribose conjugates whose contents increased more than 1.5 folds in urines of cancer patients compared to healthy controls. (B) The identified ribose conjugates whose contents decreased more than 1.5 folds in urines of cancer patients compared to healthy controls. Each number represents one compound listed in Table 2. And the numbers listed in the overlap region between cycles represent the common compounds that either increased (A) or decreased (B) in different cancer types compared to healthy controls.

We found that different contents of ribose conjugates were associated with different types of cancers (Table 2). The variable pattern of ribose conjugates in patients with various kinds of cancer may be due to the heterogeneity of different cancers. Nevertheless, 4 compounds, 5′-deoxy-5′-methylthioadensine, N^4-acetylcytidine, 1-ribosyl-*N*-propionylhistamine and N^2,N^2,7-trimethylguanosine, were found more than 1.5 folds increase in urines of all the examined types of cancers, which may be employed as potential indicator for the screening of cancers. From a clinical standpoint, the information contained in the human urine should provide clinicians and clinical chemists with a convenient, centralized resource from which to learn more about human urine and its unique chemical constituents. And additional research should provide an insight into the better use of urinary nucleosides as indicators of cancers.

Methods

Reagents. Fused-silica capillary (250 μm i.d. × 360 μm o.d.) was purchased from Yongnian Optic Fiber Plant (Hebei, China). Tetramethoxysilane (TMOS) and 3-mercaptopropyltrimethoxysilane (MPTMS) were purchased from Wuhan University Silicone New Material (Wuhan, China). Azobisisobutyronitrile (AIBN) and poly(ethylene glycol) with the molecular weight of 6000 (PEG-6000) were all purchased from Shanghai Chemical Reagent Corporation (Shanghai, China). AIBN was purified by recrystallization from ethanol at 40°C. 3-acrylamidophenylboronic acid (AAPBA) and creatinine were purchased from Sigma-Aldrich (Beijing, China). Organic solvents were all of HPLC grade. The water used throughout all experiments was purified using a Milli-Q apparatus (Millipore, Bradford, USA). All other reagents were obtained from various commercial sources and were of analytical grade unless otherwise indicated.

2′-Deoxycytidine (dC), 2′-deoxyguanosine (dG), 2′-deoxyadenosine (dA), thymidine (T), cytidine (rC), guanosine (rG), adenosine (rA), uridine (rU), 1-methyladenosine, N^6-methyladenosine, 5′-deoxyadenosine, inosine, xanthosine, 3-methylcytidine, N^4-acetylcytidine, 5-methyluridine, 3-methyluridine, pseudouridine, double hydrogen zeatin-riboside (DHzR) were purchased from Sigma-Aldrich (Beijing, China). The standard solution of each analyte was prepared at 1.0 mg/mL in H_2O and stored at −20°C.

Urine samples. The urine samples from 10 lung cancer patients, 10 colorectal cancer patients, 10 nasopharyngeal cancer patients and 10 healthy controls were collected

from Hubei Cancer Hospital, China. Detailed information can be found in Table S1 in Supporting Information. Healthy controls were selected based on medical history and physical examination. All the patients were diagnosed with cancer for the first time and had not been given any treatment at the time point of urine samples collection. The healthy controls and cancer patients were not detected with other diseases. Written informed consent was obtained from the study subjects, and an approval was granted by the Hubei Cancer Hospital Ethics Committee and met the declaration of Helsinki. All the experiments were performed in accordance with Hubei Cancer Hospital Ethics Committee's guidelines and regulations.

Urine sample collection and pretreatment were performed according to previous report[38,39] and the detailed procedure can be found in Supporting Information.

Preparation of BOHCMC. To activate the silanol groups, the fused-silica capillaries were sequentially washed with 1 M NaOH for 2 h, H_2O for 30 min, 1 M HCl for 1 h, H_2O for 30 min and methanol for 30 min followed by drying under nitrogen flow at 160°C for 6 h. For the preparation of BOHCMC, a polymerization mixture containing acetic acid (HAc) (0.01 M, 500 mg), PEG-6000 (45 mg), TMOS (185 mg), MPTMS (15 mg), AAPBA (15 mg) and AIBN (1 mg) was completely mixed and degassed by ultra-sonication for 5 min. The mixture was then manually introduced into the activated fused silica capillary (250 μm i.d. × 360 μm o.d.) by a syringe. After both ends of the capillary were sealed with two pieces of silicone rubber, the mixture was incubated at 40°C for 12 h for simultaneous polymerization and "thiol-ene" click reaction. The resulting monolith was completely flushed with water and ACN sequentially to remove the PEG-6000 and other residuals.

Characterization of BOHCMC. The specific surface area of prepared boronate-affinity organic-silica hybrid monolithic materials were measured by nitrogen adsorption-desorption experiments using a JW-BK specific surface area and pore size analyzer (JWGB Sci& Tech Co., Ltd., Beijing, China). Before measurement, the monolithic cubic pieces were evacuated in vacuum and heated to 120°C for 4 h to remove the physically adsorbed substances. Specific surface area values were determined by the Brunauer-Emmett-Teller (BET) equation at P/P_0 between 0.05 and 0.3[40]. The microscopic morphology of the monoliths was examined by scanning electron microscopy (SEM) using a Quanta 200 scanning electron microscope (FEI Company, Holland).

Boronate-affinity monolithic capillary liquid chromatography. The boronate-affinity monolithic capillary liquid chromatography experiments were performed on a SHIMADZU capillary LC system consisting of a Shimadzu LC-20AB binary pump (Tokyo, Japan), one FVC nano valve of two positions (Tokyo, Japan), a 5 μL sample loop, one GL Sciences MU 701 UV-vis detector with a 6 nL detection cell (Tokyo, Japan). To achieve micro-flow rate of 5 μL/min for separation, a T-union with one end connected to the FVC nano valve and the other end connected to a capillary (10 cm-long, 250 μm i.d.) was employed as a flow splitter between the pump and FVC nano valve. All the experiments were performed at 25°C. The chromatograms were recorded at a wavelength of 254 nm. Mobile phase A consisted of 20 mM ammonium formate (pH 8.0). Mobile phase B was a mixture of 0.1% formic acid in water. The mobile phase gradient was 0–6 min, 0% B, 6–15 min, 100% B.

Evaluation of the stability and extraction capacity of BOHCMC. To evaluate the stability, the BOHCMC was used three times per week for four weeks. After each analysis, the column was rinsed by water for 30 min and then stored at 4°C. To evaluate the extraction capacity, the extraction equilibrium profile was assessed by increasing the sample injection volume according to previously described method[41].

On-line SPME-LC-MS/MS. The on-line SPME-LC-MS/MS analysis system consisted of a MicrOTOF-Q orthogonal-accelerated TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with an ESI source (Turbo Ionspray) and a Shimadzu LC-20AB binary pump HPLC (Tokyo, Japan), a SIL-20AC auto sampler, and a DGU-$20A_3$ degasser (Figure 1). A 10-cm long BOHCMC (250 μm i.d. × 360 μm o.d.) was employed as the on-line SPME column. The analytical column was performed on a Waters Sunfire™ C18 column (150 mm × 1.0 mm i.d., 3.5 μm, Waters, MA) with a flow rate of 0.05 mL/min at 25°C. Formic acid in water (0.1%, v/v, solvent A) and formic acid in methanol (0.1%, v/v, solvent B) were employed as mobile phase. A gradient of 0–5 min 5% B, 5–10 min 5% to 30% B, 10–20 min 30% to 50% B, 20–60 min 50% B, and 60–70 min 5% B was used.

Data acquisition and processing were performed using Bruker Daltonics Control 3.4 and Bruker Daltonics Data analysis 4.0 software. The mixture of the nucleoside standards sample was employed to optimize the mass spectrometry conditions under positive ion mode (detailed mass spectrometry parameters can be found in Supporting Information).

Determination of *cis*-diol-containing nucleosides and ribosylated metabolites in urine. For the identification of the *cis*-diol-containing compounds in urine, a pooled sample that included urines from 10 lung cancer patients, 10 colorectal cancer patients, 10 nasopharyngeal cancer patients and 10 healthy controls was used. The pooled sample was lyophilized to dryness and then reconstituted 20 mM ammonium formate (pH 8.0). And then, 50 μL was analyzed by on-line SPME-LC-MS/MS. The prospective molecular formulas of the *cis*-diol-containing compounds were generated based on the accurate molecular mass, MS/MS fragment information and isotope patterns of elemental composition using Bruker Daltonics Data analysis 4.0 software. A mass tolerance of 5.0 mDa was set and a maximum elemental composition of C = 50, H = 100, N = 50, O = 50, S = 10 was used. The molecular formulas and MS/MS fragment information obtained by TOF were further searched in the database of METLIN (http://metlin.scripps.edu) for putative identification. As for the relative quantification of *cis*-diol-containing compounds between cancer patients and healthy controls, four pooled samples from three types of cancers as well as healthy controls were made. And then each pooled sample from one type of cancer patients was compared to the pooled sample of healthy controls.

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Author contributions

B.F.Y., Y.Q.F. and H.P.J. conceived and designed the research, analyzed the data and wrote the paper; H.P.J., C.B.Q., J.M.C. performed the research; C.B.Q. collected the urine samples.

Additional information

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