Hindawi Publishing Corporation
Journal of Chemistry
Volume 2014, Article ID 616459, 6 pages
http://dx.doi.org/10.1155/2014/616459

Research Article

Synthesis and *In Vitro* and *In Vivo* Evaluation of a New ^{68}Ga-Semicarbazone Complex: Potential PET Radiopharmaceutical for Tumor Imaging

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Received 30 December 2013; Revised 20 July 2014; Accepted 5 August 2014; Published 8 September 2014

Academic Editor: Doina Humelnicu

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In an attempt to develop new tumor imaging radiotracers with favorable biochemical properties, we have synthesized new ^{68}Ga-2-acetylpyridine semicarbazone (^{68}Ga-[APSC]$_2$) as a potential positron emission tomography (PET) tumor imaging agent using a straightforward and a one-step simple reaction. Radiochemical yield and purity were quantitative without HPLC purification. Biodistribution studies in nude mice model bearing human MDA-MB-231 cell line xenografts displayed significant tumor uptake of ^{68}Ga-[APSC]$_2$ radiotracer after 2 h postinjection (p.i.). The initial results demonstrate that ^{68}Ga-[APSC]$_2$ radiotracer may be useful probe for detecting and staging of hypoxic tumor using PET imaging modality.

1. Introduction

The physical properties of the positron emitter gallium-68 (^{68}Ga) and its availability from ^{68}Ge/^{68}Ga generator, together with the well-known coordination chemistry of gallium, make it one of the most attractive radionuclides for PET imaging. Therefore, varieties of ^{68}Ga-labeled bioactive molecules for potential use beyond the measurement of glucose metabolism were developed and investigated [1–3]. This development will certainly help nuclear medicine centers without the on-site availability of a cyclotron and consequently patient care improvement.

Heterocyclic thiosemicarbazones, semicarbazones, and their metal complexes have been extensively investigated as potential antitumor agents [4–12]. Most studied thiosemicarbazones and semicarbazones were the pyridine-based compounds which might be attributed to their resemblance to pyridoxal metabolites that attached to coenzyme B6-dependent enzymes and caused enzyme inhibition [4, 11]. Varieties of metallic PET and single photon emission computed tomography (SPECT) radionuclides were incorporated into thiosemicarbazones chelates. Among these radionuclides, $^{67/68}$Ga and copper-62/64 ($^{62/64}$Cu) have shown considerable success for targeting hypoxic and normoxic tumor xenografts in athymic mouse models [12–19].

Recently, radiolabeled ^{67}Ga-thiosemicarbazone complex was synthesized with high radiochemical yield and purity. Biodistribution study in fibrosarcoma bearing mice revealed specific tumor accumulation after 2 h postinjection (p.i.) which may suggest that ^{68}Ga is a better candidate for tumor imaging than ^{67}Ga [20].

Due to the well-known coordination chemistry of gallium, high stability of Ga^{III}-semicarbazone in comparison with its thiosemicarbazone complexes [4], and its enhanced

antineoplastic activity, we were interested in developing new radiotracers with favorable biochemical properties; we here report the synthesis, characterization, and preclinical evaluation of new ^{68}Ga-2-acetylpyridine semicarbazone (^{68}Ga-[APSC]$_2$) as a potential PET imaging agent for hypoxic tumor.

2. Experimental

2.1. Materials and Measurements. The chemicals used in the study were all of analytical reagent grade, were purchased from Aldrich, and were used without further purification unless stated. Commercial ^{68}Ga-generator based on a SnO_2 phase adsorbing was obtained from iThemba Lab, South Africa. ^{67}Ga was produced by the bombardment of enriched zinc-68 (^{68}Zn) targets (1 g ± 10%) with 30 MeV protons from the Cyclone 30 cyclotron internal beam using the ^{68}Zn (p, 2n) ^{67}Ga nuclear process. Sep-Pak cartridges were purchased from Waters-Millipore. TLC-SG sheets were purchased from Grace Sciences Inc. High Performance Liquid Chromatography (HPLC) analysis was carried out on Luna, Phenomenex C-18 reversed phase column (analytical, 250 mm × 4.6 mm). The solvent system used was isocratic (eluent: ACN/H_2O 95/5, with 0.1% TFA at flow rate of 1.0 mL/min). A Jasco chromatographic system equipped with a variable wavelength ultraviolet monitor and in tandem with a Canberra flow through radioactivity detector was used. Ultraviolet absorption was monitored at 254 nm. Chromatograms were acquired and analyzed using BORWIN software. Melting points were determined on a Thomas-Hoover Unimelt capillary melting point apparatus. Mass spectroscopy was run on Quattra electrospray mass spectrometer (ES-MS). Elemental analyses were performed on a Perkin Elmer CHN 2400 analyzer. IR bands were recorded on a Perkin Elmer spectrophotometer 1000 in the spectral range 200–4000 cm^{-1} with sample in the form of KBr pellets. ^{1}H-^{13}C NMR spectra were obtained in DMSO-D_6 on JEOL NMR 400 MHz. Log K was calculated by potentiometric methods as described in the literature [21].

2.2. 2-Acetylpyridine Semicarbazone (APSC). APSC compound was synthesized using method reported previously [22]. In brief, aqueous solution (20 mL) of semicarbazide hydrochloride (1.11 g, 1.0 mmol) was added to aqueous solution (20 mL) of 2-acetyl pyridine (1.20 g, 1.0 mmol) in the presence of sodium acetate (1.36 gm, 1.0 mmol). The reaction mixture was stirred vigorously for 2 h. The crystalline white product was filtered off and washed with ether. Yield: 96%, mp = 188–192°C. [$C_8H_{10}N_4O$] elemental analysis (calculated): C: 54.0 (53.9%), H: 5.8 (5.7%), and N: 31.2 (31.4%). IR (υ_{max}/cm^{-1}): (NH: 3473); (NH_2: 3375); (CH_3: 2280); (C=O_{as}: 1686); (C=O_{sy}: 1579); (C=N: 1443); (C–O: 1104); (heterocyclic bases: 771, 626, 561, and 448). NMR (^{1}H, ppm, DMSO-d6): 3.39 (3H, CH_3); 7.3 (2H, NH_2); 7.3 (1H, CH=C); 7.7 (1H, CH=C); 8.3 (1H, CH–C); 8.5 (1H, CH–N); 9.5 (1H, NH). (^{13}C-ppm, DMSO-d6): 12 (CH_3); 120.7 (C–H); 123.8 (C–HC); 136 (C–H); 145 (C=N); 148 (C–HD); 155 (C–N=C); 157 (C=O). m/z (ESI$^+$) 179 ([M]$^+$).

2.3. 2-Acetylpyridine Semicarbazone Ga-Complex (Ga-[APSC]$_2$). Ga-[APSC]$_2$ complex was prepared according to previously published method with slight modification [22]. To the ligand APSC (0.019 g, 0.106 mmol), dissolved in hot ethanol (EtOH, 10 mL), was added $GaCl_3$ in EtOH solution (1 mL, 0.05 mmol) drop-wise. The reaction mixture was refluxed for 2 h under N_2 atmosphere with vigorous stirring. The reaction mixture cooled down to room temperature and kept under N_2 atmosphere until the yellow-white product precipitated. Precipitate was then filtered and washed with ether. Yield: 66%. M.P. = 207°C. [$C_{16}H_{20}N_8O_2Cl$ Ga, Mr 461] elemental analysis (calculated): C: 41.3 (41.6%), H: 4.6 (4.3%), N: 24.4 (24.3%), and Cl 15.56 (15.1%). IR (υ_{max}/cm^{-1}): (NH, 3444); (NH_2: 3245); (CH_3: 2365); (C=O_{as}: 1678); (C=N: 1528); (C–O: 1066); (Ga-N: 540); (Ga-O: 470), 3114 (C–H). NMR (^{1}H, ppm, DMSO-d6): 1.08 (3H, CH_3); 7.3 (2H, NH_2); 7.6 (1H, CH=C); 8.1 (1H, CH=C); 8.3 (1H, CH–C); 8.7 (1H, CH–N); 9.8 (1H, NH). (^{13}C-ppm, DMSO-d6): 15 (CH_3); 120.7 (CH–C); 123.8 (C=C); 136 (C=C); 146 (C=N); 148 (C–N); 157 (C–N=C); 65 (C–O). m/z (ESI$^+$) 427 ([M]$^+$). Log K = 22.

2.4. Radiochemistry. ^{68}Ge/^{68}Ga generator was eluted with suprapure HCl (0.6 M, 6 mL) in 0.5 mL fractions. The two fractions with the highest $^{68}GaCl_3$ (37–300 MBq) activity were generally used for labeling purposes [23, 24]. The ^{68}Ga-[APSC]$_2$ complex was prepared by the addition of APSC (50 μg) to a screw-cap vial containing $^{68}GaCl_3$ (185–300 MBq) dissolved in 0.1% acetic acid/methanol (AcOH/MeOH, v/v; pH = 4.5). The reaction mixture was heated for 30 min at 90°C and cooled to room temperature, followed by the dilution with normal saline, and the product was passed via a 0.22 μm membrane filter to be ready for injection. The same procedure was repeated with ^{67}Ga-[APSC]$_2$.

2.5. Stability in Plasma. For stability in plasma, ^{68}Ga-[APSC]$_2$ complex (100 μL, 20 μCi each) was incubated with human plasma (500 μL) in duplicate at 37°C for 1–4 h. This was followed by precipitation using a mixture of ACN/EtOH (400 μL, 1/1 v/v) and centrifugation at 5000 rpm for 5 min. The supernatant layer was then analyzed by HPLC under the conditions described above.

2.6. In Vivo Biodistribution and Tumor Targeting. Approval for the animal protocol used in this study was obtained from the Institutional Animal Care and Use Committee. Animal biodistribution experiments were performed according to the international regulations governing the safe and humane use of laboratory animals in research [25]. MDA-MB-231 breast cancer cell line is known to be induced by hypoxic tumor cells [26]. Human MDA-MB-231 xenograft mouse models were used for *in vivo* tumor targeting experiments. For the implantation of tumor xenografts, approximately 3 × 10^6 MDA-MB-231 cells in suspension of 100 μL sterile saline

were injected subcutaneously into the right thigh of each mouse as reported in the literature [27]. Tumors were allowed to grow for 2-3 weeks in which tumors had reached weights of ~500 mg. Mice were injected *via* the lateral tail vein with 100 μL of the radiotracers formulated in saline. Each dose contained ~20 μCi (740 kBq) of the ^{68}Ga-[APSC]$_2$ radiotracer. Animals were sacrificed at different time intervals (4 mice at each interval) and tissues of interest were dissected, weighed, and assayed for radioactivity. The percentage of the injected dose per gram (% ID/g) was then calculated by counting all tissues in a γ-well counter using a stored sample of the injection solution as a standard to estimate the total dose injected per mouse.

2.7. Statistical Analysis. Data are expressed as mean ± S.D. where appropriate. For data comparisons, Student's t-test was performed for the mean values using Graph-Pad Software (Graph-Pad Software Inc., San Diego, CA, USA). A probability value of $P < .05$ was considered statistically significant.

3. Results and Discussion

3.1. Organic Chemistry. The one-step synthesis of the monosemicarbazones proceeds by the reaction of 2-acetylpyridine and semicarbazides in the presence of acetic acid in aqueous media. Similarly, the reaction of APSC with $GaCl_3$ has furnished Ga-[APSC]$_2$ complex in good yield as shown in Scheme 1. Elemental analysis measurements for APSC ligand and Ga-[APSC]$_2$ reference complex were found to correspond to the calculated results. Chemical purity of Ga-[APSC]$_2$ complex was found to be greater than 99% as represented by a single peak in the HPLC chromatogram with 12.5 min retention time (Figure 1).

The IR bands in the region 3160–3440 cm^{-1}, attributed to the symmetrical stretching mode $\upsilon(NH_2)$ in the spectra of the ligand, were shifted to 3460–3210 cm^{-1} in those of the complex. The coordination of the ligand APSC$^-$ via the azomethine nitrogen is reflected by the shift of υ(C=N) band at 1443 cm^{-1} to higher frequency at 1528 cm^{-1} for Ga-[APSC]$_2$ complex and complexation viathe C=O strong bands at 1678 cm^{-1} and 1579 cm^{-1} was attributed to υ(C=O) asy-, symmetrical, respectively. The υ(CN) band for APSC$^-$ also shifted to higher frequency at 1497 cm^{-1} for the Ga-[APSC]$_2$ complex, indicating coordination of the ligand viathe pyridine nitrogen atom. Medium intensity bands at 540 and 470 cm^{-1} were assigned to stretching vibrations υ(Ga-N) and υ(Ga-O), respectively, whereas weak bands at 771, 626, and 561 cm^{-1} were assigned to heterocyclic ring.

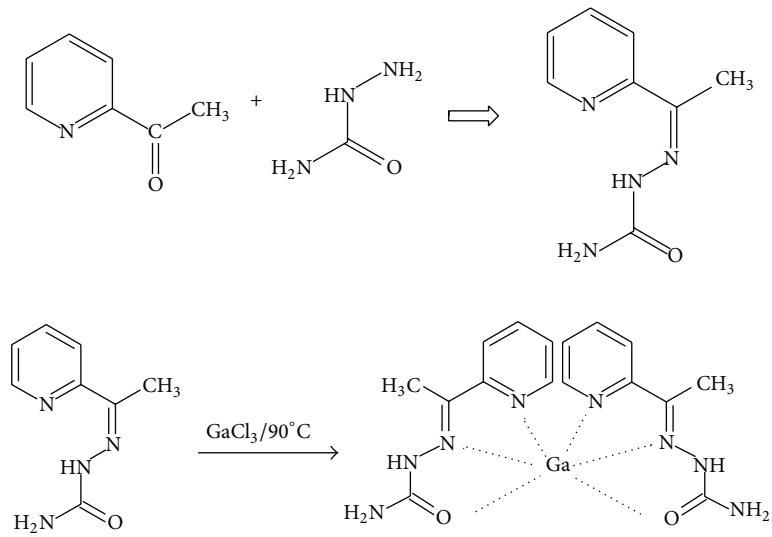
The NMR spectra of the ligand APSC$^-$ and its Ga(III) complex were recorded in DMSO-d6. The coordination of two tridentate monoanionic ligands to Ga(III) through oxygen and azomethine nitrogen atoms resulted in upfield shifts of two quaternary carbon atoms bound to oxygen atoms from 157 ppm for APS to 65 for Ga-[APSC]$_2$ and downfield shifts of the methyl carbons bound to the azomethine groups from 12.15 ppm for APS$^-$ to 15.16 and 14.18 ppm for the pyridine rings of Ga-[APSC]$_2$. The carbons *ortho* to the nitrogen atom were shifted upfield correspondingly by 8.1 and 7.7 ppm for C1 and C2 and 7.3 and 8.5 ppm for C3 and C4, which indicates involvement of the pyridine nitrogen atoms in coordination with Ga(III).

The ES mass spectrum of Ga-[APSC]$_2$ complex recorded in the positive mode showed a 100% relative intensity (R.I.) peak at m/z 427 which corresponds to the theoretical isotopic distribution excluding chloride counter ion.

3.2. Radiochemistry. Due to the well-known coordination chemistry of Ga(III), high stability of Ga(III)-semicarbazone complexes, and their enhanced antineoplastic activity and in an attempt to develop new radiotracers with favorable biochemical properties for hypoxic tumor imaging, we synthesize $^{67/68}$Ga-[APSC]$_2$ as a potential PET and SPECT tumor imaging agent. The synthetic approaches for the preparation of ^{68}Ga-APSC chelates were simple and straightforward and gave ^{68}Ga-[APSC]$_2$ complexes in quantitative radiochemical yields and purities as assessed by TLC and HPLC in less than 60 min. The R_f values for $^{68}GaCl_3$ and ^{68}Ga-[APSC]$_2$ complex were 0.0 and 0.9, respectively (Figure 2), and the retention times for the same compounds were 4.12 and 12.70 min, respectively (Figure 3), which correspond to the reference sample (Figure 1).

3.3. Stability in Plasma. The proteolytic degradation of the ^{68}Ga-[APSC]$_2$ complex was determined in human plasma *in vitro*. The proteolytic degradation of the ^{68}Ga-[APSC]$_2$ complex revealed that this complex remained sufficiently stable (>95%) during incubation at 37°C for at least 1 h and then started degrading to 75 and almost 50% after 2 and 4 h of incubation, respectively.

3.4. In Vivo Biodistribution and Tumor Uptake. The biodistribution data in nude mice bearing human MDA-MB-231 cell line xenografts at 1, 2, and 4 h p.i. ($n = 4$ for each time point) for ^{68}Ga-[APSC]$_2$ radiotracer and 24 h p.i. for ^{67}Ga-[APSC]$_2$ are shown in Table 1. The biodistribution data indicate that the radiolabeled compound showed high initial uptake in the blood that reduced with time. At 4 h p.i., only 3.45% ID/g was presented in the blood circulation. The uptake in the major organs such as the liver, lungs, heart, spleen, and kidneys was also high at 1 and 2 h p.i. but radioactivity was cleared by these organs and no significant retention of radioactivity was observed at 24 h p.i., with the exception of kidneys which retained somewhat high radioactivity (up to 6.15% ID/g) at 24 h p.i. The uptake in the tumor was somewhat moderate at 1 h p.i but reached the maximum (11.58 ± 1.07% ID/g) at 2 h p.i. At 4 and 24 h p.i., 4.08% ID/g and 2.22% ID/g of radioactivity, respectively, were found in the tumor indicating a good retention of the radioactivity by the MDA-MB-231 tumors. The tumor-to-muscle ratio obtained at 2 h p.i. was 7.52. It is worth mentioning here that the tumor uptake value obtained with this radiotracer is found to be similar to the uptake profile reported earlier for ^{67}Ga-thiosemicarbazone [20] and the tumor uptake washed out after 24 h p.i. indicating the opposite to the free ^{68}Ga(III) cation that usually



Scheme 1: Synthesis of 2-actylpyridine semicarbazone (APSC) and Ga-2-acetylpyridine semicarbazone (Ga-$[APSC]_2$) complexes (Ga = $^{nat/67/68}$ Ga).

Table 1: Biodistribution of ^{68}Ga-(2-acetylpyridine semicarbazone)$_2$ complex (^{68}Ga-$[APSC]_2$) in tumor bearing nude mice.

	1 h	2 h	4 h	24 h*
Blood	17.71 ± 1.33	6.41 ± 0.10	3.45 ± 0.57	2.13 ± 0.08
Liver	7.04 ± 0.06	4.14 ± 0.31	3.51 ± 0.09	2.13 ± 0.53
Lung	7.34 ± 0.37	4.03 ± 0.27	3.61 ± 0.72	1.87 ± 0.20
Kidney	6.18 ± 0.94	4.81 ± 0.47	3.25 ± 0.21	6.15 ± 0.72
Intestine	5.21 ± 0.44	8.22 ± 0.02	3.80 ± 0.89	3.76 ± 0.68
Heart	5.75 ± 0.92	2.31 ± 0.55	2.16 ± 0.27	1.07 ± 0.08
Muscle	2.33 ± 0.33	1.54 ± 0.49	0.75 ± 0.11	0.37 ± 0.08
Spleen	4.76 ± 0.43	5.01 ± 0.80	3.88 ± 0.24	2.88 ± 0.26
Tumor	3.52 ± 0.59	11.58 ± 1.07	4.08 ± 0.04	2.22 ± 0.01

The values are average of % injected dose/gram ± SD for $n = 4$.
*Animals were injected with ^{67}Ga-$[APSC]_2$ radiotracer.

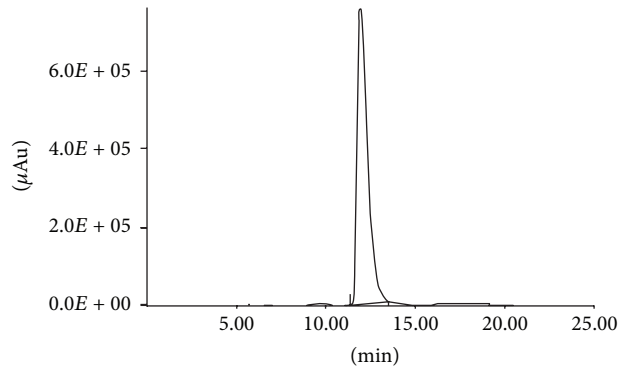


Figure 1: HPLC chromatogram of Ga-(2-acetylpyridine semicarbazone)$_2$ complex (Ga-$[APSC]_2$).

increased with time. However, the accumulation of ^{68}Ga-$[APSC]_2$ tracer in most of the other organs revealed that it is at least twofold lower than the tracer ^{67}Ga-thiosemicarbazone [20]. Thus, the good tumor targeting capabilities together with the favorable biodistribution profile of ^{68}Ga-$[APSC]_2$ warrant further evaluation and may tempt one to infer that this PET radiotracer may be useful as a molecular probe for detecting and staging of hypoxic tumors and their metastasis as well as monitoring tumor response to treatment.

4. Conclusion

^{68}Ga-$[APSC]_2$ was synthesized in quantitative radiochemical yield and purity in less than 60 min. Biodistribution results of ^{68}Ga-$[APSC]_2$ in nude mice model bearing human MDA-MB-231 cell line xenografts demonstrated significant tumor uptake at 2 h p.i. and favorable pharmacokinetics over previously investigated ^{67}Ga-$[APSC]_2$ radiotracer. These results demonstrate that ^{68}Ga-$[APSC]_2$ radiotracer may be useful as PET probe for detecting and staging of hypoxic tumor; however, further evaluation is warranted.

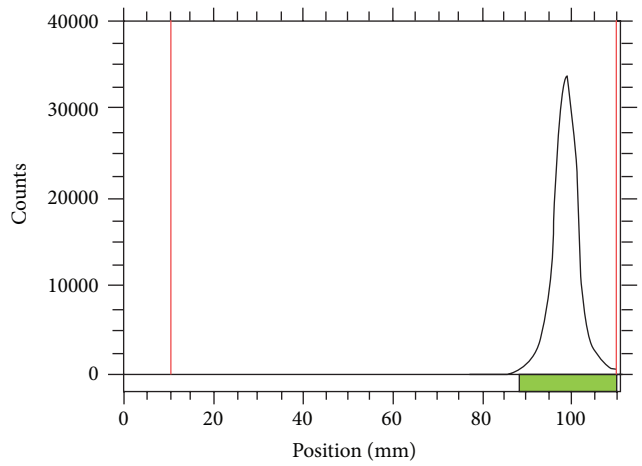


Figure 2: TLC chromatogram of ^{68}Ga-(2-acetylpyridine semicarbazone)$_2$ complex (^{68}Ga-[APSC]$_2$).

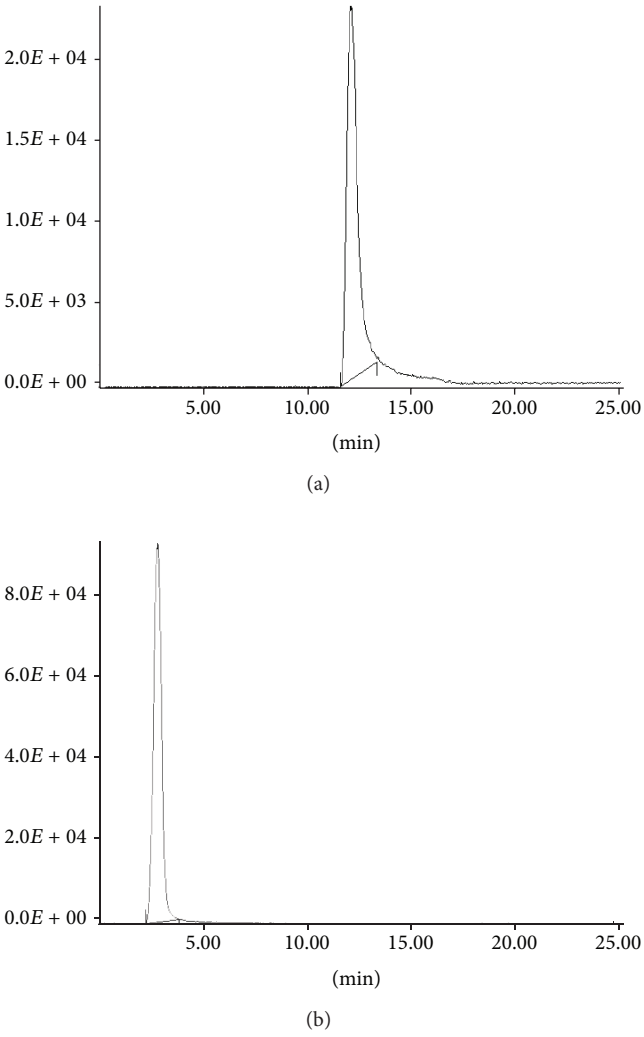


Figure 3: HPLC chromatograms of ^{68}Ga-(2-acetylpyridine semicarbazone)$_2$ complex (^{68}Ga-[APSC]$_2$) (a) and ^{68}GaCl$_3$ (b).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The research team would like to acknowledge the King Faisal Specialist Hospital and Research Center (KFSH&RC) and the International Atomic Energy Agency (IAEA) for their support. This research project was partially supported by a grant from the "Research Center of the Center for Female Scientific and Medical Colleges," Deanship of Scientific Research, King Saud University. Also, special thanks are due to the comparative medicine department for their support in animal studies.

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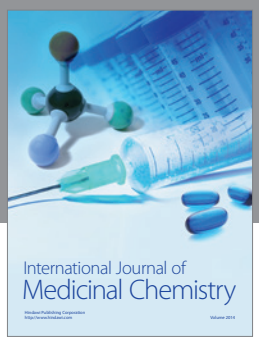

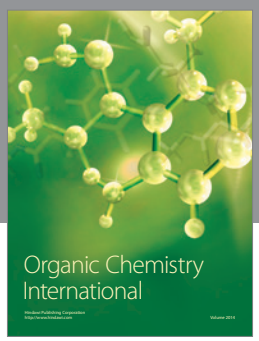

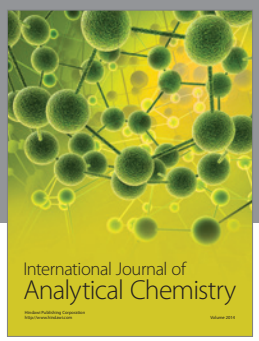

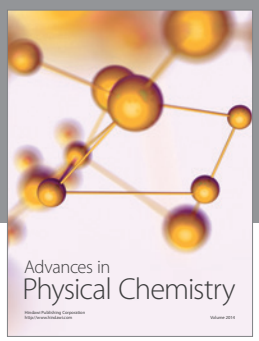

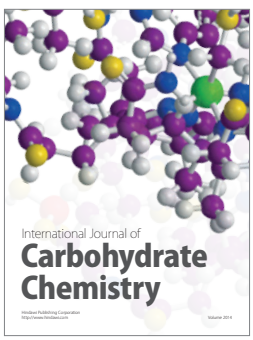

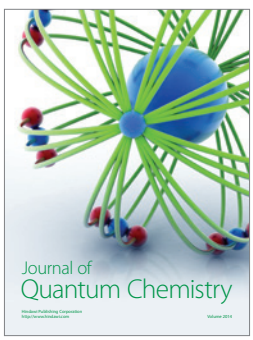

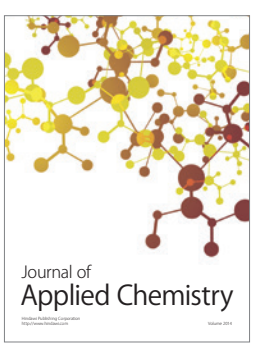

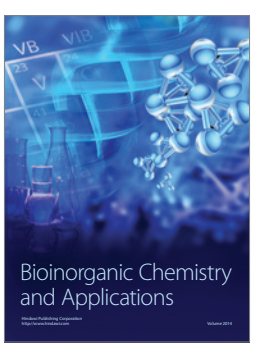

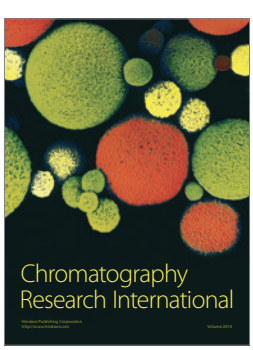

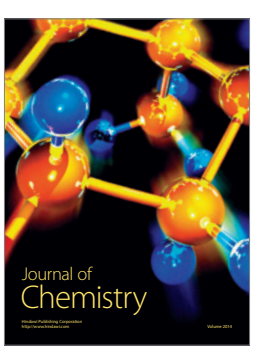

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