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A rapid and simple assay to determine the blood and urine concentrations of 1-(5-[$^{123/125}$I]iodo-5-deoxyarabinofuranosyl)-2-nitroimidazole, a hypoxic cell marker[1]

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Abstract

Pharmacokinetic and dosimetric parameters of the hypoxic tissue imaging agent iodoazomycin arabinoside (^{123}I-IAZA) have been investigated in human volunteers. In conjunction with this study it was necessary to develop an assay for low levels of the radiolabelled compound in blood and urine. A combination of high-performance liquid chromatography (HPLC) and gamma counting produced a highly selective, sensitive and rapid assay for the analysis of $^{123/125}$I-IAZA in human and animal blood and urine samples. Conventional HPLC assays for the tracer quantities of this radioactive agent in blood have not been reported previously. The addition of non-radiolabelled IAZA to the blood and urine samples containing radiolabelled IAZA allowed the pharmaceutical to serve as its own internal standard. This reverse isotope dilution approach permitted identification of the appropriate HPLC peak by UV detection, followed by highly sensitive quantification of the radiolabelled species by gamma counting. Blood samples were prepared for HPLC by a solid-phase extraction without the loss of IAZA from serum, with an extraction efficiency of $99.7 \pm 7.1\%$ from human serum. Urine samples could be analyzed directly by HPLC, without the solid-phase extraction step. The detection limit in biological fluids depends on the specific activity of radiolabelled $^{123/125}$I-IAZA. In this study it was possible to detect serum concentrations of ^{123}I-IAZA as low as 7.46 pg (21 fmol) per ml. The radiometric detection limit for ^{123}I-IAZA in this assay was 10.8 Bq ml^{-1} of serum.

Keywords: Radiometric assay; HPLC assay; IAZA; Blood; Urine; Quantification of radiolabelled compounds

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1. Introduction

Iodoazomycin arabinoside (IAZA) (Fig. 1) is a sugar-coupled 2-nitroimidazole which has been developed collaboratively at the University of Al-

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berta and the Cross Cancer Institute, Edmonton [1]. It is selectively trapped in hypoxic cells, undergoing reductive metabolism to reactive species that bind to cellular macromolecules, thereby preventing reverse diffusion of the tracer. Since tissue hypoxia is a consequence of numerous disease conditions including cancer [2], diabetes [3], rheumatoid arthritis [4], cardiac insufficiencies [5] and stroke [6], there is demand for a non-invasive diagnostic imaging agent, such as radioiodinated IAZA (^{123}I-IAZA), which could differentiate viable but hypoxic tissue from dead tissue. ^{123}I-IAZA has already been administered to more than sixty cancer patients in an ongoing limited clinical study [7]. Nuclear medicine imaging of these patients has permitted assessment of the hypoxic status of the tumors. The ^{125}I-labelled analog (^{125}I-IAZA) has found wide application in in vitro studies and in preclinical studies with research animals.

In its standard clinical application IAZA is administered to humans in a very low chemical dose (1–10 mg typical) and with levels of radioactivity suitable for nuclear medicine imaging (185 MBq of ^{123}I typical). The challenge was to develop a sensitive and reproducible assay which would allow the detection of unchanged IAZA in blood and urine. The drug levels in blood rapidly fall below the level of UV detection in high-performance liquid chromatography (HPLC). In addition, the radioactivity levels become too low for detection by standard HPLC flow-through radiometric detectors.

A semi-quantitative analysis of radioiodinated ^{123}I-IAZA was reported previously, based on measurement of total radioactivity in serial blood samples taken from cancer patients. These data were used to estimate the pharmacokinetics of ^{123}I-IAZA [2]. Counting the blood samples for total radioactivity is a common method of pharmacokinetic analysis of radiolabelled agents [8]. However, this scintigraphic method is a nonspecific assay which cannot distinguish between ^{123}I-IAZA and its ^{123}I-labelled metabolites. In practice both total blood activity, as determined by direct scintillation counting of blood, and selective counting of $^{123/125}$I-IAZA as described in the present assay, are clinically relevant. While ^{123}I-IAZA alone is metabolically trapped in hypoxic tissues, total radioactivity from ^{123}I-IAZA and its radioactive metabolites in blood and surrounding tissues constitutes background interference which must be reduced before clear scintigraphic tissue images can be obtained. It is therefore important to distinguish between intact $^{123/125}$I-IAZA and radiolabelled metabolites including free radioiodine produced by deiodination of the radiopharmaceutical. Consequently, a very specific assay which combines the selectivity of HPLC and the sensitivity of gamma counting has now been developed.

This assay has been used, with minor modifications, to determine the concentration of ^{125}I-IAZA in mouse [9] and rat [D. Stypinski et al., unpublished data], and ^{123}I-IAZA in human blood samples [D. Stypinski et al., unpublished data]. A similar reverse isotope dilution technique [10] has been developed to determine the concentration of $^{123/125}$I-IAZA in urine of rats and human volunteers.

2. Experimental

2.1. Reagents

IAZA was synthesized and radiolabelled by an exchange reaction with Na[^{123}I]I or Na[^{125}I]I, as described previously [1]. Water (Caledon Laboratories, Georgetown, Canada) and methanol

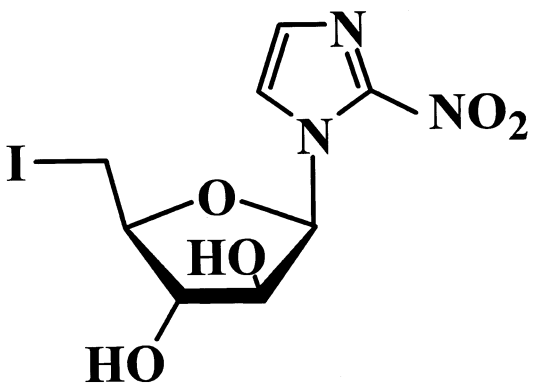

Fig. 1. Structure of 1-(5-iodo-5-deoxy-β-D-arabinofuranosyl)-2-nitroimidazole (IAZA).

(Fisher Scientific, Montreal, Canada) were HPLC grade. Sterile normal saline (NS) was supplied by Baxter Corporation (Toronto, Canada) in 100-ml i.v. bags.

2.2. Blood sample collection and preparation

Prior to the collection of the blood samples, the internal standard (cold IAZA; 30 μg in 250 μl normal saline) was added to the collection tubes. In the animal studies the collection tubes were Fisherbrand® 1.5-ml microcentrifuge tubes (Fisher Scientific), while for human studies 9-ml SST Vacutainer® blood collection tubes (Becton Dickenson) were used. To prevent clotting of blood, heparin sodium (Hepalean®, Organon Teknika) was used in the animal studies. Clot formation and centrifugation in the absence of heparin sodium were used for human blood samples in order to speed up sample preparation. The tubes were weighed before and after blood sample collection to determine the blood volume.

After coagulation, the human blood samples were centrifuged (15 min at $1528 \times g$) in a Beckman Model TJ-6 Centrifuge. A small aliquot of serum (50–80 μl) was withdrawn for the determination of total radioactivity in the serum fraction. The remainder of the serum was separated from the clot for further analysis, while the clot was counted for total radioactivity. Rat blood samples were centrifuged for 5 min in a Brinkman Model 5412 centrifuge, with packed cells and the supernatant separately counted for total radioactivity. Mouse blood samples were not centrifuged, instead whole blood was counted to determine total radioactivity. All human blood samples were handled using a protocol approved by the Biosafety Committee, University of Alberta, and clinical samples were obtained through a protocol approved by the Medical Ethics Committee of the Cross Cancer Institute.

2.3. Solid phase extraction

Disposable Waters™ SepPak cartridges (C18, Classic, Short body) were used for cleanup and isolation of $^{123/125}$I-IAZA in human serum, rat plasma, and mouse whole blood samples. Each cartridge was first conditioned with methanol (4 ml) followed by water (5 ml). Immediately thereafter, the samples were manually loaded onto the cartridge with a 1- or 5-ml syringe, depending on sample size. Water (3 ml) was pushed through the cartridge, effectively eluting most of the unwanted components. This eluent was discarded, and methanol (2 ml) was passed through the cartridge. This methanol fraction, which contained $^{123/125}$I-IAZA, was collected for further analysis. In the animal studies, where ^{125}I-IAZA (^{125}I decay $t_{1/2} =$ 60 days) was used, these samples were evaporated to dryness and reconstituted with normal saline (250 μl). For human samples where ^{123}I-IAZA (^{123}I decay $t_{1/2} = 13.2$ h) was used, the methanol eluent fractions were collected into 3 ml serum collection Vacutainer® tubes that had been precalibrated to allow for the determination of the volume of the eluent, thereby bypassing the evaporation and reconstitution steps. All of the samples were analyzed using HPLC.

2.4. Urine sample preparation

Aliquots of urine (5 ml) were first counted for total radioactivity. Prior to HPLC analysis, aliquots (1 ml) were filtered through a sterile Acrodisc® 0.2-μm low protein binding disposable filter to remove any possible particulate matter, spiked with several micrograms of cold IAZA, and injected onto the HPLC directly without any dilution.

2.5. Instrumentation and chromatographic conditions

The HPLC system consisted of a U6K manual injector (Waters), a single M45 pump (Waters), and a scanning ultraviolet detector (Waters Millipore Lambda-Max Model 401 LC Spectrophotometer). The eluent from the HPLC was collected using a fraction collector (FRAC-100, Pharmacia). The chromatographic data acquisition was performed with IBM-compatible PC with Baseline 810 data processing software (Waters, Mississagua, Canada).

The mobile phase used for blood sample analysis was a pre-mixed, degassed water/methanol so-

Table 1
Extraction efficiency values for IAZA in the matricies studied

	Whole blood	Serum	Plasma
Human	0.369 ± 0.036 (5–10 ml) ($n = 25$)	0.997 ± 0.071 (2.5–5 ml) ($n = 7$)	ND[a]
Rat	0.564 ± 0.055 (0.2–0.5 ml) ($n = 6$)	ND	0.823 ± 0.013 (0.1–0.25 ml) ($n = 4$)
Mouse	0.462 ± 0.106 (0.75–1.0 ml) ($n = 2$)	ND	ND

[a] ND, not determined for reasons explained in the text.

lution (3:2, v/v), and for urine sample analysis pre-mixed, degassed water/methanol solution (7:3, v/v). In both cases the flow rate was 2 ml min^{-1}. The chromatographic separation was achieved using a reverse-phase analytical column (Waters, μBondpack Radial-Pak C18 Cartridge). The detector was set at $\lambda = 325$ nm, which is the λ_{max} of IAZA as determined by PU 8740 UV/VIS scanning spectrophotometer (Phillips). All samples were analyzed in triplicate. HPLC system stability was tested at the beginning, during and at the end of each day by injecting standard IAZA in normal saline.

For radiometry, HPLC serial eluent fractions were collected with careful recovery of the eluent volume that contained the IAZA peak as indicated by the UV detector. The duration of sample collection was 1.25 min (blood) and 1.65 min (urine) corresponding to a sample volume of 2.5 ml and 3.3 ml, respectively. All HPLC samples were counted for radioactivity using a Gamma 8000 (Beckman) gamma scintillation counter. Counter background radioactivity was determined by counting several empty sample vials along with each sample set.

2.6. Extraction efficiency

A calibration plot of UV absorption against IAZA amount was established by analyzing 25-μl samples of five different IAZA concentrations over the 15–100 μg ml^{-1} range. All the samples were analyzed in triplicate.

Whole blood extraction efficiency studies were performed with blank blood samples (no IAZA dose to the donor) collected from rat and human volunteers. Cold IAZA internal standard (30 μg in 250 μl of normal saline) was mixed in vitro with donor whole blood and subsequently analyzed using the solid phase extraction procedure and HPLC analysis as described above. In addition, plasma (rats) and serum (human) extraction efficiencies were determined in a similar manner for blank rat plasma and human serum, by adding the internal standard to each, followed by solid phase extraction and HPLC analysis. The exact volumes of each matrix are given in Table 1.

The extraction efficiency from all of the matrices was calculated by comparing the HPLC UV absorbance peak of the internal standard in normal saline versus that determined for the internal standard added to the matrix and extracted using the SepPak extraction procedure, normalized for any dilution.

3. Results and discussion

The calibration curve of IAZA was found to be linear over the 0.375–2.50 μg (1.06–7.04 nmol) range, with a calculated regression line with a slope of 0.01596, $f(0) = -0.02073$; the correlation coefficient (r^2) was 0.99. The calibration curve of the internal standard IAZA was used to determine the extraction efficiency. It is important to note that the extraction efficiency is independent of the amount of $^{123/125}$I-IAZA in the samples. For each assay, 30 μg of internal standard IAZA was added to the matrix and therefore the contribution of the radiolabelled IAZA to the total IAZA was insignificant. Under our experimental conditions, even for samples taken at 1 min after $^{123/125}$I-IAZA administration, the $^{123/125}$I-IAZA concentration in the samples accounted for less than 1.0% of the total of sample radiolabelled plus standard IAZA. Consequently, the extraction effi-

ciency of any sample will depend only on the sample matrix.

It was determined that samples of IAZA in plasma were stable for at least one month when stored either refrigerated or frozen (data not shown). Furthermore, there was no difference in the efficiency of extraction of IAZA from plasma samples stored for 24 h at room temperature when compared to extraction from samples stored at refrigerator temperature. For in vivo studies samples were routinely refrigerated within 30 min of collection and brought to room temperature only at the time of analysis. The final volume of each MeOH fraction, following SepPak extraction, was adjusted to 2 ml and, therefore, the samples were always analyzed in the linear range of the calibration curve (1.11 ± 0.11 mg 200 ml^{-1} injection for a $36.9 \pm 3.6\%$ extraction efficiency).

The results of the extraction efficiency studies are summarized in Table 1. With the exception of the mouse blood samples, all relative standard deviation values were $< 10\%$. For the mouse, whole blood samples were loaded directly onto the SepPak cartridge, and the presence of cellular debris may have introduced greater variability to the assay. Since rat plasma samples were less than one tenth the size of the human serum samples, the lower extraction efficiency from the rat plasma may reflect sample loss during sample transfers rather than sample loss during the SepPak extraction procedure; however, the wash eluent from rat plasma has not been analyzed to verify this observation. Similarly, the greater extraction efficiency value from the rat whole blood reflects the volume expansion of the supernatant once the internal standard, in 250 μl of normal saline, was added to whole blood (200–500 μl for rats, 5–10 μl for human). The low extraction efficiency from the whole blood in humans suggests that almost 63% of the analyte was retained in the clot. This has been confirmed by collecting blood samples from seven volunteers within 1–3 min of ^{123}I-IAZA administration, and counting the clot and the serum. The radioactivity in the clot was $62.6 \pm 1.4\%$ of total sample radioactivity. In addition, for one of the volunteers, the water wash, the SepPak cartridge and the volume not accommodated by the 1.0-ml hold-up volume of the cartridge were collected. All of the activity in the serum sample was accounted for, with negligible loss to the SepPak cartridge and the volume of serum not accommodated by the cartridge. The percent radioactivity in the water wash was negligible initially, but increased with time after administration of the radiopharmaceutical. This increase was most likely due to the presence of radiolabelled water soluble metabolites of ^{123}I-IAZA.

A representative chromatogram and radiochromatogram of a human blood sample is shown in Fig. 2. ^{123}I-IAZA from the blood samples along with the added IAZA internal standard eluted at 5.98 min. The presence of the internal standard allowed UV detection of the appropriate fraction containing ^{123}I-IAZA and provided a guide for the collection of the eluent for subsequent gamma counting. The chromatograms from mouse and

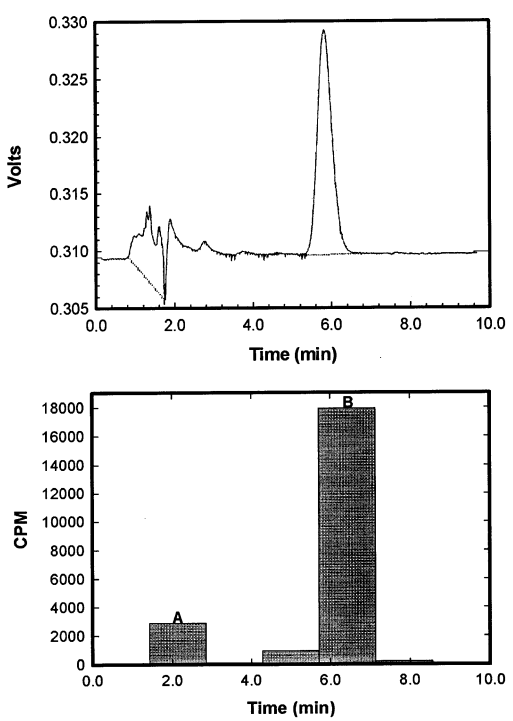

Fig. 2. HPLC chromatogram (upper panel) and corresponding radiometric plot (lower panel) of a representative human blood sample after the administration of a single i.v. dose of ^{123}I-IAZA. In the lower panel, A represents polar radioactive metabolites, while B represents radioactive *I-IAZA.

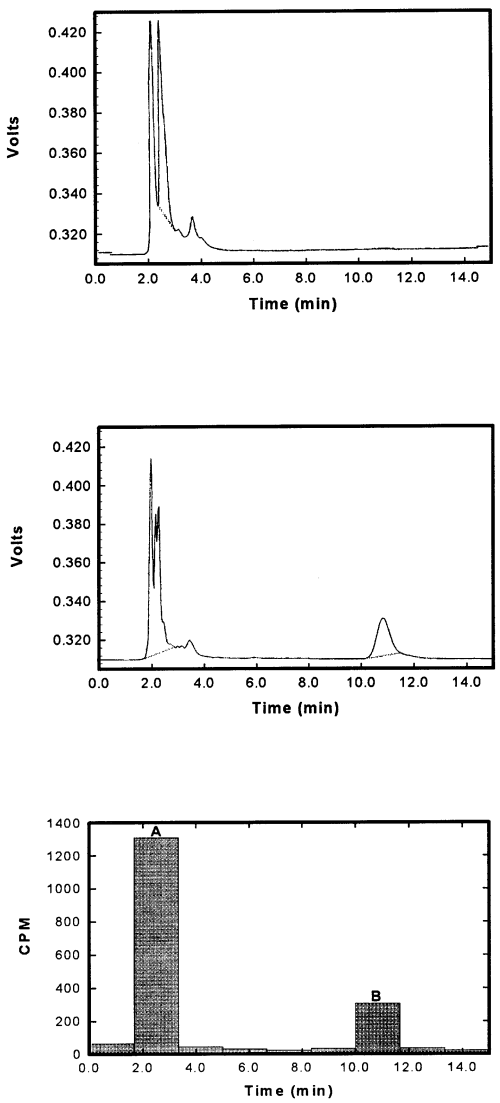

Fig. 3. HPLC chromatograms of representative cumulative 28-h human urine sample without the internal standard (upper panel) and after addition of 25 μg ml^{-1} of the internal standard (middle panel), and a corresponding radiometric plot (lowest panel) after the administration of a single i.v. dose of ^{123}I-IAZA. In the lowest panel, A represents polar radioactive metabolites, while B represents radioactive *I-IAZA.

rat blood samples containing ^{125}I-IAZA and the internal standard were similar to those in Fig. 2.

A representative chromatogram and radiochromatogram of a human urine sample is shown in Fig. 3. In the case of urine, cold IAZA was added just prior to the HPLC injection, and served only to identify the location of the ^{123}I-IAZA peak. The retention time of IAZA in urine when urine was injected directly onto the HPLC was 11.16 min. It was necessary to increase the retention time of IAZA in urine samples to improve the separation of this peak from the interfering UV peak from an endogenous component of urine which overlapped the IAZA peak when the solvent and flow conditions described for blood were used. The calculation of the amount of ^{123}I-IAZA in urine samples was accomplished by determining the radioactivity due to the ^{123}I-IAZA in a known volume of sample, correcting for radioactive decay from the time of ^{123}I-IAZA administration to the subject and multiplying by the total volume of urine collected. Rat urine containing ^{125}I-IAZA produced similar chromatograms.

Decay corrections were made based on first-order decay of ^{123}I, using the equation $A = A_0 \exp(-0.693t/t_{1/2})$, where A is the activity at the time of measurement (in the gamma counter), A_0 is the activity at the time of sample collection from the patient, $t_{1/2}$ is the 13-h physical decay half-life of ^{123}I, and t is the time elapsed between sample collection and measurement. Since ^{125}I has a 60-day decay half-life and since same-day processing and counting of samples was used, no decay correction was necessary for these samples.

The minimum detection limit of this assay depends not so much on the chemical amount of the agent in the blood, as on the specific activity of the radiolabelled compound (MBq mg^{-1}). In the human studies, for example, the specific activity of the administered ^{123}I-IAZA dose varied by more than tenfold, with a nominal dose of 185 MBq of radioactivity administered in anywhere from 0.1 to 1.2 mg of cold IAZA carrier [D. Stypinski et al., unpublished data]. As a result, the minimum detection limit of the assay for ^{123}I-IAZA, as determined from the last sample of the volunteer who received the smallest chemical dose (0.1 mg), was 7.46 pg (21 fmol) per ml of serum. The actual detection limit for this assay is determined by the detection limit for radioactive counting. In the human blood sample analysis this limit was set arbitrarily at three times the back-

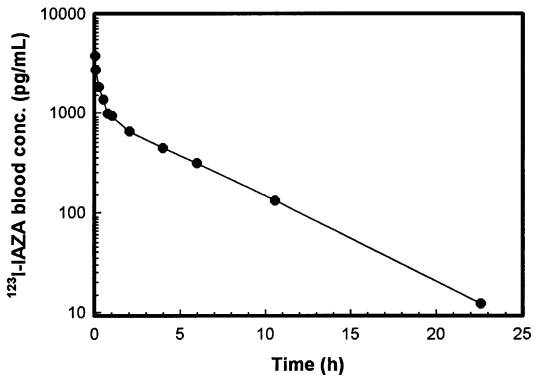

Fig. 4. Representative blood concentration vs. time plot from a human volunteer after a single i.v. dose of ^{123}I-IAZA.

ground count rate. The background [38.2 ± 11.0 CPM] was determined from a blank blood sample collected from each volunteer before the administration of ^{123}I-IAZA. Since the counting efficiency of the gamma counter for ^{123}I was 0.4, this cut-off count rate [38.2 ± 11.0 CPM] corresponded to a detection limit of about 645 DPM ml^{-1} (10.8 Bq ml^{-1}) of serum. In general, this assay was sensitive enough to trace activity up to the point where greater than 99.0% of the predicted area under the concentration versus time curve had been accounted for in all volunteers who received ^{123}I-IAZA [D. Stypinski et al., unpublished data].

The detection limit for ^{125}I-IAZA was similar; ^{125}I analysis has the advantages of practically no loss of counts from radioactive decay during processing (decay half-life for ^{125}I is 60 days) and better counting efficiency of the gamma counter (> 0.75).

4. Conclusions

The HPLC/radiometric assay described above, based on the principles of reverse isotope dilution, has been successfully applied to the analysis of hundreds of blood and urine samples from humans, rats and mice. A representative blood ^{123}I-IAZA profile in one of the volunteers is shown in Fig. 4. The assay has proven to be selective, sensitive, and rapid, allowing the analysis of a large number of samples in a relatively short time period. The minimum radioactive detection limit for ^{123}I-IAZA was 10.8 Bq ml^{-1} of serum, with the molar detection limit determined by the specific activity of the radiolabelled $^{125/123}$I-IAZA.

Acknowledgements

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