Cancer Chemother Pharmacol (2006) 57: 335–342
DOI 10.1007/s00280-005-0060-1

ORIGINAL ARTICLE

M. W. DeGregorio · K. H. Dingley · G. T. Wurz
E. Ubick · K. W. Turteltaub

Accelerator mass spectrometry allows for cellular quantification of doxorubicin at femtomolar concentrations

Received: 23 March 2005 / Accepted: 30 May 2005 / Published online: 27 July 2005

Abstract Accelerator mass spectrometry (AMS) is a highly sensitive analytical methodology used to quantify the content of radioisotopes, such as ^{14}C, in a sample. The primary goals of this work were to demonstrate the utility of AMS in determining total cellular [^{14}C]anthracycline concentrations following administration of doxorubicin (DOX) and to develop a sensitive assay that is superior to high performance liquid chromatography (HPLC) for the quantification of [^{14}C]anthracycline at the tumor level. In order to validate the sensitivity of AMS versus HPLC with fluorescence detection, we performed three studies comparing the cellular accumulation of DOX: one in vitro cell line study, and two in vivo xenograft mouse studies. Using AMS, we quantified cellular [^{14}C]anthracycline content up to 4 h following in vitro exposure at concentrations ranging from 0.2 pg/ml (345 fM) to 2 μg/ml (3.45 μM) [^{14}C]DOX. The results of this study show that, compared to standard fluorescence-based HPLC, the AMS method was over five orders of magnitude more sensitive. Two in vivo studies compared the sensitivity of AMS to HPLC using a nude mouse xenograft model in which breast cancer cells were implanted subcutaneously. After sufficiently large tumors formed, [^{14}C]DOX was administered intravenously at two dose levels. Additionally, we tested the AMS method in a nude mouse xenograft model of multidrug resistance (MDR) in which each mouse was implanted with both wild type and MDR+ cells on opposite flanks. The results of the second and third studies showed that [^{14}C]anthracycline concentrations were significantly higher in the wild type tumors compared to the MDR+ tumors, consistent with the MDR model. Although this method does not discriminate between parent drug and metabolites, the extreme sensitivity of AMS should facilitate similar studies in humans to establish target site drug delivery and to potentially determine the optimal treatment dose and regimen.

Keywords Accelerator mass spectrometry · Doxorubicin · Multidrug resistance · Breast cancer

M. W. DeGregorio (✉) · G. T. Wurz
University of California, Davis, Department of Internal Medicine, Division of Hematology and Oncology, Cancer Center, 4501 X Street Room 3016, Sacramento, CA, 95817, USA
E-mail: mwdegregorio@ucdavis.edu
Tel.: +1-916-7342360
Fax: +1-916-7342374

K. H. Dingley · E. Ubick · K. W. Turteltaub
Biological Sciences Directorate, Lawrence Livermore National Laboratory, 7000 East Avenue, Livermore, CA, 94551, USA

Introduction

The aim of this work is to demonstrate accelerator mass spectrometry (AMS) as a highly sensitive methodology for quantifying drug concentrations in tumor cells, with the ultimate goal of using this methodology to quantify drug delivery to target tissues in humans. Development of a sensitive and quantitative method of determining drug concentrations in tumors using aspirated cells or needle biopsies would therefore be clinically useful for guiding cancer therapy on an individual basis.

Current approaches to obtaining pharmacokinetic data in tumors include non-invasive techniques such as positron emission tomography (PET) [13, 20, 21] and nuclear magnetic resonance (NMR) [18]. Naturally, ^{19}F NMR requires either that a fluorine atom already be an integral part of the drug of interest, e.g., 5-fluorouracil, or the incorporation of fluorine into the drug molecule. This method, while highly useful, is also compromised by lack of sensitivity, with a detection limit in the range of 0.01–0.1 mmol for fluorinated drugs. Deuterium (^{2}H) NMR has also been used, but it has an effective sensitivity about 10- to 100-fold less than ^{19}F NMR [14]. Positron emission tomography is a powerful imaging tool for the quantification of positron emitting radiotracers and can provide very detailed spatiotemporal data without biopsy sampling. Several labeled substrates

for MDR-related drug transporters have been synthesized for imaging of the MDR phenotype in tumors [13, 20, 21]. However, PET's main limitations are the short half-life of the isotopes used and the specialized equipment and syntheses required [23].

Other more sensitive methods have been developed which require tissue collection for analysis ex-vivo. These include HPLC and capillary electrophoresis with either fluorescence or UV detection, as well as mass spectrometry and enzyme immunoassays [5, 7, 8, 12]. The most widely used methodology for the quantification of DOX is HPLC with fluorescence detection. A method has been developed for the determination of DOX and its metabolite doxorubicinol in plasma, with lower limits of quantification of 1.0 (1,724 pM) and 0.50 ng/ml (862 pM) plasma, respectively [7]. The main drawback of these methods is that they require a relatively large amount of material for analysis and are therefore not sufficiently sensitive for quantification of DOX in aspirated cells or needle biopsies. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), one of the most sensitive analytical methods developed to date, has demonstrated sensitivity similar to the above methods when analyzing patient plasma [11, 17, 19] or tumors [22]. However, much greater sensitivity, in the zeptomole (10^{-21} mol) range, has been reported using CE-LIF for the determination of DOX and metabolites in cell extracts and subcellular fractions [1, 2].

In contrast to these more traditional techniques for drug quantification, AMS is a relatively new biomedical technology for quantification of isotope-labeled compounds in biological samples with attomole (10^{-18} mol) or greater sensitivity and high precision. The high sensitivity of AMS translates to the use of low chemical and radioisotope doses and relatively small sample sizes, which enables tracer studies to be performed safely in humans, using exposures which are environmentally or therapeutically relevant, whilst generating little radioactive waste. Most biomedical AMS studies completed to date have employed ^{14}C as the radiolabel, although the capability exists for detecting other isotopes including ^{3}H, ^{26}Al, ^{41}Ca, ^{10}Be, ^{36}Cl, ^{59}Ni, ^{63}Ni and ^{129}I [24].

The goal of this work was to demonstrate that AMS has sensitivity advantages over HPLC with traditional fluorescence detection and offers equivalent sensitivity to the most sensitive CE-LIF methods for the quantification of DOX in biological samples.

Materials and methods

Chemicals

[^{14}C]Doxorubicin hydrochloride (55 mCi/mmol) was obtained from Amersham Pharmacia Biotech UK Limited. If radio or chemical purity was less than 98%, it was purified by HPLC prior to use. Conditions were 1 ml/min flow rate using a mobile phase consisting of 50% acetonitrile/35% water/15% 0.1 M phosphoric acid (v/v) using a C18 column and absorbance monitoring at 487 nm. The retention time of DOX was approximately 8 min. Doxorubicin hydrochloride, which was used as an HPLC standard, for the radiolabeled DOX, and also in the medium of MDA-MB-231-A1 cells, was obtained from Adria Laboratories (Columbus, OH, USA). Trypsin (2.5%) was obtained from Invitrogen (Carlsbad, CA, USA), and powdered PBS and EDTA were purchased from Sigma Chemical Company (St. Louis, MO, USA). Doxorubicin HCl (mw 580.0) and daunorubicin HCl (DNR; mw 564.0) used in the HPLC analysis of DOX in the cells and tissues were purchased from Sigma. All reagents (chloroform, methanol, water, acetonitrile) were purchased from Fisher Scientific (Pittsburgh, PA, USA) and were of HPLC grade.

Laboratory animals

The study was approved by the Institutional Animal Care and Use Committee at Lawrence Livermore National Laboratory in accordance with the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female, BalbC, Nu/Nu athymic nude mice (4–6 weeks of age) were purchased from Simonsen Laboratories (Gilroy, CA, USA) and were housed five per cage in negative airflow caging (Lab Products one-cage micro-isolator system) on autoclaved wood chip bedding. Cages and water bottles were autoclaved prior to use. Mice were fed autoclaved 18% protein diet (Harlan, Indianapolis, IN, USA) and sterile filtered acidified water ad libitum.

Cell culture

Wild type MDA-MB-231, obtained from the American Type Culture Collection (Manassas, VA, USA), and multidrug resistant MDA-MB-231-A1, obtained from the late Dr. William McGuire, estrogen receptor-negative human breast cancer cells were grown under normal conditions (37°C, 5% CO_2/95% air) in improved minimum essential medium (IMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) in 75 cm^2 filter-capped flasks (Corning, Corning, NY, USA). In addition, the medium of the MDA-MB-231-A1 cells contained 2 μg/ml DOX to maintain selective pressure. Cells were subcultured on a weekly basis. At each passage, the spent medium was aspirated, followed by the addition of warm (37°C) 0.25% trypsin solution (80% 1X PBS, 10% 10 mM EDTA, 10% 10X trypsin). Flasks remained at room temperature while the cells detached, after which the appropriate medium, warmed to 37°C, was added to the flasks. Aliquots of the cell slurry were then added to new flasks containing the appropriate medium. One passage prior to an experiment, the selective pressure on the MDA-MB-231-A1 cells was removed.

Time course of cellular DOX accumulation in cell culture

To assess the kinetics of cellular accumulation, MDA-MB-231 or MDA-MB-231-A1 cells were exposed to [^{14}C]DOX (specific activity 0.000171 mCi/mmol) at a concentration of 2 μg/ml (3.45 μM). Cells were plated in T-75 flasks, three flasks for each time-point and cell line. Cells were plated at a density of 50,000 cells/ml and allowed to grow to near confluency in a total volume of 15 ml medium. One milliliter of a 32-μg/ml stock solution of [^{14}C]DOX, prepared in sterile 0.9% sodium chloride, was then added to each flask, yielding a final concentration of 2 μg/ml. Cells were incubated for 10 min, 30 min, 2 h or 4 h. One additional flask of each cell line was used as a negative control and was spiked with 1 ml of a 0.9% sodium chloride solution. Following the exposure, the culture medium was aspirated, and the cells were washed with 5 ml of ice-cold (4°C) PBS. The rinse was then aspirated and 3 ml of fresh ice-cold PBS were added. The cells were removed from the flasks by scraping, and the resulting cell suspensions were transferred to clean glass tubes. Each tube was gently vortexed and a 0.2-ml aliquot was removed for counting. A Coulter Model ZM counter (Beckman-Coulter; Miami, FL, USA) was used for cell counts. The cell samples were split equally to produce replicates for both AMS and HPLC analysis.

Dose-response of cellular DOX accumulation in cell culture

MDA-MB-231 or MDA-MB-231-A1 cells were exposed to [^{14}C]DOX for 4 h at concentrations of 0 pg/ml, 0.14 pg/ml (0.24 pM), 0.47 pg/ml (0.81 pM), 5 pg/ml (8.62 pM), 100 pg/ml (172.4 pM), 1 ng/ml (1.72 nM) and 2 μg/ml (3.45 μM; specific activities from 0.00012 mCi/mmol to 53 mCi/mmol). Cells were plated in T-150 flasks, one flask for each concentration and cell line. Cells were plated at a density of 100,000/ml and allowed to grow to near confluency in a total volume of 25 ml medium. Concentrated stock solutions of [^{14}C]DOX were prepared at 3.64 pg/ml, 12.22 pg/ml, 130 pg/ml, 2.6 ng/ml, 26 ng/ml, and 52 μg/ml, and 1 ml of each was added to the appropriate flasks to yield the desired final concentrations. Cells were incubated for 4 h, harvested and counted. Each cell sample was then split into six replicates to produce three samples for AMS and three for HPLC analysis.

Cellular DOX accumulation in a xenograft model

Dose-response for DOX accumulation in MDA-MB-231 tumor cells

Female, athymic, BalbC Nu/Nu nude mice (4–6 weeks of age) were implanted with MDA-MB-231 cells. Using a 1-ml tuberculin syringe fitted with a 21-gauge needle, each mouse was subcutaneously inoculated with approximately 10.6 million cells (injection volume 200 μl) on the right front flank.

Three weeks later, the animals were systematically divided into two groups (eight animals per group) which exhibited equal mean body weights. The groups were randomly assigned to the treatments. One additional group of four animals received no DOX and acted as controls. [^{14}C]DOX was administered intravenously via a tail vein using a 28-gauge needle. A dose of 0.1 or 1 mg/kg body-weight [^{14}C]DOX contained in a volume of 50 μl was administered to each mouse. The specific activities were 0.1057 and 0.0106 mCi/mmol for the 0.1 and 1 mg/kg body-weight doses, respectively. Two hours after dosing, each mouse was euthanized by CO_2 asphyxiation. Blood was collected by cardiac puncture into tubes containing heparin, as anticoagulant, and centrifuged at 3,000×*g* for 10 min, to separate the plasma. Liver and tumors were excised promptly, weighed, and then snap-frozen in liquid nitrogen. All samples were stored at −80°C until analysis. Samples were analyzed by AMS and HPLC to determine the tissue drug levels, which were used as the basis for assessing whether the drug was sufficiently delivered.

Comparison of DOX accumulation in MDA-MB-231 and MDA-MB-231-A1 tumor cells

Female, athymic, BalbC Nu/Nu nude mice (4–6 weeks of age) were implanted with MDA-MB-231 and MDA-MB-231-A1 cells on opposite flanks. Each mouse was subcutaneously inoculated with approximately 2.88 million cells/200 μl (MDA-MB-231) and 3.43 million cells/200 μl (MDA-MB-231-A1). Two weeks later, the animals were injected intravenously via tail vein as described above with 1 mg/kg body-weight [^{14}C]DOX (specific activity 0.0192 mCi/mmol). Two animals were used as controls and received sterile 0.9% saline only. Two hours later, tumors were removed, weighed, and snap frozen in liquid nitrogen until analysis by AMS.

AMS analysis

Cell samples (each between 107,000–3,010,000 cells) and plasma (10 μl) were added to quartz tubes and up to 1 mg of tributyrin carrier was added to provide sufficient total carbon for AMS. Five to ten milligrams of liver or tumor from each animal was analyzed without the addition of carrier. Each tumor sample was homogenized prior to analysis to establish a homogeneous distribution of DOX and its metabolites. Samples were prepared for AMS analysis and the $^{14}C/^{13}C$ ratios measured using standard procedures which are described in [14, 15, 24]. The ratios were converted to ng anthracycline/10^6 cells or ng anthracycline/g tissue following the subtraction of the ^{14}C contribution from any added tributyrin and control samples. AMS measures

only the amount of ^{14}C in a sample as an isotope ratio and provides no structural information, and thus no distinction between DOX metabolites and parent compound within a sample can be made.

HPLC analysis

Chromatographic conditions

The HPLC system consisted of a Beckman (Fullerton, CA, USA) Model 320 gradient liquid chromatograph, a Model 420 controller, and two Model 110A pumps. The system was equipped with a Beckman 5-μm reverse phase C18 Ultrasphere ODS 4.6×250 mm column, 100-μl injection loop, and a Rheodyne (Rohnert Park, CA, USA) injector. The column was maintained at room temperature (20–25°C) for all analyses. DOX and internal standard (IS) were detected with a Linear Instruments Model LC305 fluorescence detector (Thermo Separation Products, Inc., San Jose, CA, USA) set at an excitation wavelength of 470 nm and an emission wavelength of 550 nm. A Hewlett-Packard (Corvallis, OR, USA) Model 3394 integrator was used for recording retention times and peak heights. The mobile phase consisted of acetonitrile (ACN)/water/0.1 M H_3PO_4 50/35/15 (v/v) and was degassed under vacuum for approximately 20 min daily prior to column equilibration. Before sample analysis each day, the column was allowed to equilibrate for approximately 45 min at a flow rate of 1.0 ml/min. Upon completion of daily sample analyses, the column was washed sequentially with ACN/water 50/50 (v/v) and methanol/water 50/50 (v/v) for 20 min each.

Sample preparation, extraction, and analysis

Prior to extraction, cell samples from the DOX cellular accumulation studies were spiked with 20 μl of a 100-μg/ml solution of DNR in saline (delivering 2 μg DNR) as internal standard. The tumor and liver samples from the xenograft study were weighed on an analytical balance, spiked with IS, and then homogenized prior to extraction. To extract, 9.0 ml of a chloroform/methanol solution (80/20, v/v) were added to all samples, which were contained in glass 16×125 mm tubes (Corning). All samples were vortexed for 1 min and then centrifuged at approximately 1,500×*g* for 15 min. The organic layer of each sample was removed by pipette, placed in a clean 16×125 mm tube, and then evaporated to dryness under a gentle stream of nitrogen in an analytical evaporator at 37°C. Samples were stored at −20°C until they could be analyzed by HPLC.

Concentrated calibration standard stock solutions were prepared in normal saline (0.9% NaCl) at the following concentrations: 250; 500; 1,000; 2,000; 4,000; 8,000; and 10,000 ng/ml. Calibration standards for analysis were prepared by spiking 100 μl of each stock solution into 1.0 ml aliquots of plasma. After spiking with IS, extraction was carried out as described above. When not is use, all standard solutions were kept frozen at −20°C in the dark.

To analyze, samples and standards were reconstituted in 200 μl mobile phase, centrifuged at 10,000×*g* for 1 min to remove particulates, and then injected onto the HPLC column. Samples were eluted isocratically at a flow rate of 1.0 ml/min. Retention times and peak heights were recorded. Final sample concentrations of DOX were calculated based on the standard calibration curve and expressed in ng/10^6 cells or ng/g tissue as appropriate.

Statistical analyses

Statistical analyses, including *t*-tests, were performed using Microsoft Excel.

Results

Time course of cellular DOX accumulation in cell culture

Doxorubicin concentrations in both MDA-MB-231 and MDA-MB-231-A1 cells in culture following exposure to [^{14}C]DOX at 2 μg/ml (3.45 μM) for 10 min to 4 h are plotted in Fig. 1. At the earliest time points, there was no significant difference in anthracycline concentrations between the two cell lines. However, by 2 h, concentrations were significantly higher ($P = 0.001$) in the MDA-MB-231 cells and continued to increase at the 4-h time point relative to the MDA-MB-231-A1 cells ($P = 0.000047$). At the 4 h time point, DOX concentrations were 4.7-fold higher in the MDA-MB-231 cells relative to the MDA-MB-231-A1 cells.

Dose-response of cellular DOX accumulation in cell culture

Anthracycline concentrations in MDA-MB-231 and MDA-MB-231-A1 cells in culture following exposure to [^{14}C]DOX for 4 h at doses in the range 0.14 pg/ml (0.24 pM) to 2 μg/ml (3.45 μM) are plotted in Fig. 2. The dose response appears to be linear over a seven orders of magnitude dynamic range, and at all doses, DOX concentrations in the MDA-MB-231 cells were significantly higher than in the MDA-MB-231-A1 cells ($P \leq 0.05$). However, as the DOX dose was reduced, the difference between the cell lines became less pronounced (2.4-fold difference at 2 μg/ml versus 1.1-fold difference at 0.14 pg/ml). Interestingly, this result suggests that the difference in accumulation of DOX can be seen at low concentrations that can be measured by AMS and not HPLC. Additional studies will address this kinetic observation.

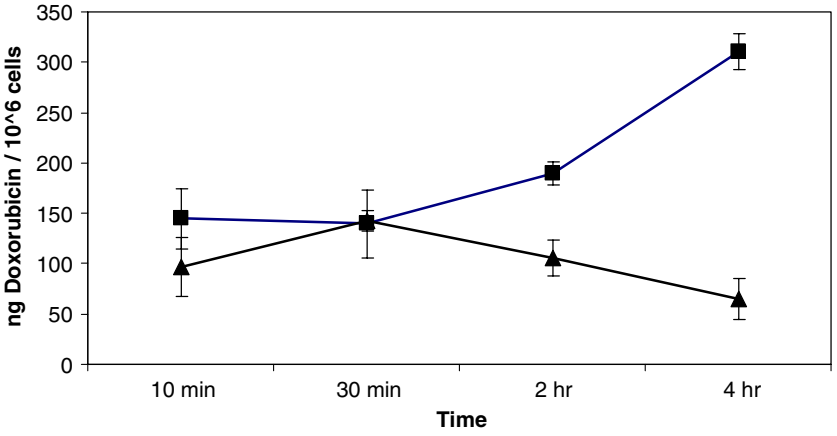

Fig. 1 Doxorubicin concentrations in MDA-MB-231 (*filled squares*) and MDA-MB-231-A1 (*filled triangles*) cells following incubation with [^{14}C]DOX for up to 4 h measured by AMS. *Error bars* represent the standard deviation of three replicate analyses per time point.

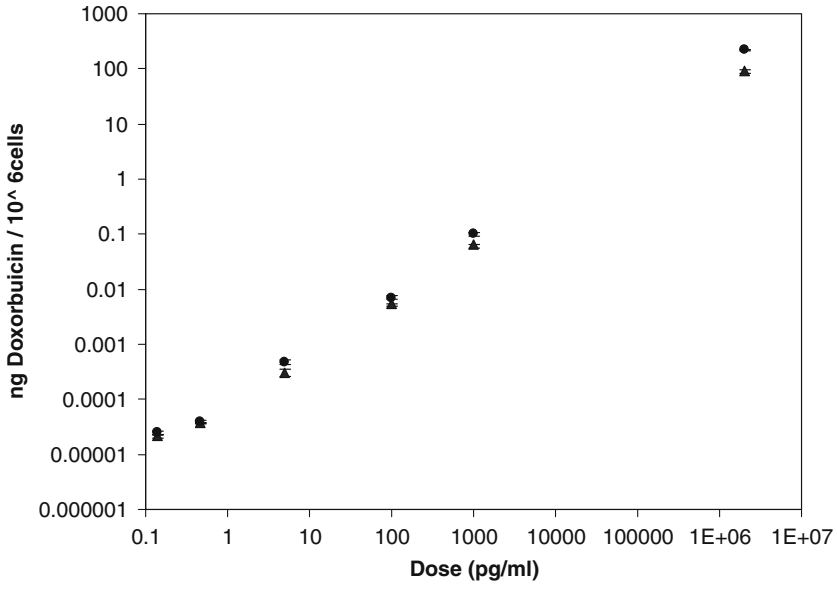

Fig. 2 Doxorubicin concentrations in MDA-MB-231 (*filled circles*) and MDA-MB-231-A1 (*filled triangles*) cells measured by AMS following incubation with [^{14}C]DOX at concentrations of 0.2 pg/ml to 2 μg/ml for 4 h. *Error bars* represent the standard deviation of three replicate analyses per dose point.

Cellular DOX accumulation in a xenograft model

Dose-response for DOX accumulation in MDA-MB-231 tumor cells

The average weight of the MDA-MB-231 tumors in the mice was 1.7 ± 0.97 g. The average concentrations of anthracycline, quantified by AMS, in the tumors, liver and plasma for both the 0.1 and 1 mg/kg body-weight doses are shown in Table 1. In all tissue types, a clear increase in anthracycline concentrations was detected with increasing dose. The highest concentration was observed in the liver, followed by the tumor tissue, and then plasma. For comparison, HPLC with fluorescence detection was used to analyze the liver and tumor tissues. The HPLC data is compared in Table 2 to that obtained following [^{14}C]anthracycline analysis by AMS in the same tissues. Plasma levels were well below the quantitative limit of the HPLC methodology (25 ng DOX/g tissue) and were not attempted. In all cases, the amounts of [^{14}C]anthracycline in the tissues measured by AMS were well above the controls (the signal to noise ratio was on average 39:1 for liver and 10:1 for tumor tissue). The HPLC with fluorescence detection technique was limited in sensitivity and in many cases was unable to detect DOX. In those samples where DOX was quantifiable by both HPLC and AMS, AMS detected on average approximately four-fold higher concentrations of [^{14}C]anthracycline.

Comparison of DOX accumulation in MDA-MB-231 and MDA-MB-231-A1 tumor cells

Seven animals that grew both MDA-MB-231 and MDA-MB-231-A1 tumors were dosed with [^{14}C]DOX. Average tumor weights were 0.093 ± 0.041 and 0.27 ± 0.11 g for MDA-MB-231 and MDA-MB-231-A1 cells, respectively. The amounts of anthracycline in the tumors, quantified by AMS, are plotted in Fig. 3. The mean concentrations of anthracycline in the MDA-MB-231 and MDA-MB-231-A1 tumors were 333.5 ± 86.2 and 230.1 ± 65.8 ng/g tissue, respectively.

Table 1 Doxorubicin concentrations in plasma and in liver and tumor tissue from female, athymic, BalbC Nu/Nu nude mice, measured by AMS, 2 h following injection with either 0.1 or 1 mg/kg [^{14}C]DOX ($N = 8$)

Dose (mg/kg)	Average tissue concentration (±SD)		
	Plasma (ng/ml)	Tumor (ng/g)	Liver (ng/g)
0.1	3.5 ± 1.6	18.8 ± 11.0	357.7 ± 110.8
1.0	40.0 ± 9.6	370.6 ± 78.6	4660.2 ± 572.4

SD Standard deviation

Table 2 A comparison of DOX concentrations in liver and tumor tissue quantified by AMS versus analysis by HPLC with fluorescence detection ($N = 8$)

Dose (mg/kg)	Average tissue concentration (±SD; ng/g)			
	Liver (AMS)	Liver (HPLC)	Tumor (AMS)	Tumor (HPLC)
0.1	357.7 ± 110.8	51.0 ± 44.5	18.8 ± 11.0	No detection
1.0	4660.2 ± 572.4	1434.0 ± 244.1	370.6 ± 78.6	50.8 ± 43.6

SD Standard deviation

The anthracycline concentrations were significantly higher in the MDA-MB-231 tumors ($P = 0.01337$). Due to the small size of the tumors, there was insufficient tissue to allow for analysis by HPLC.

Discussion

The relatively low assay sensitivity of traditional analytical methods has limited the clinical value of pharmacokinetic studies in cancer therapeutics. Traditionally, drug levels are measured in plasma or serum and non-target tissues. While there is a correlation between toxicity and plasma pharmacokinetics for at least one cancer chemotherapeutic agent, methotrexate, predicting response and toxicity and determining effective dosing of chemotherapeutic agents based upon plasma or serum pharmacokinetic data has proven to be inconclusive at best. Ideally, drug concentrations should be determined within the target tissue to more accurately assess delivery. The development of new analytical techniques such as AMS that offer greatly enhanced sensitivity over traditional HPLC has now given us the opportunity to begin serious study of tissue-specific drug delivery and kinetics.

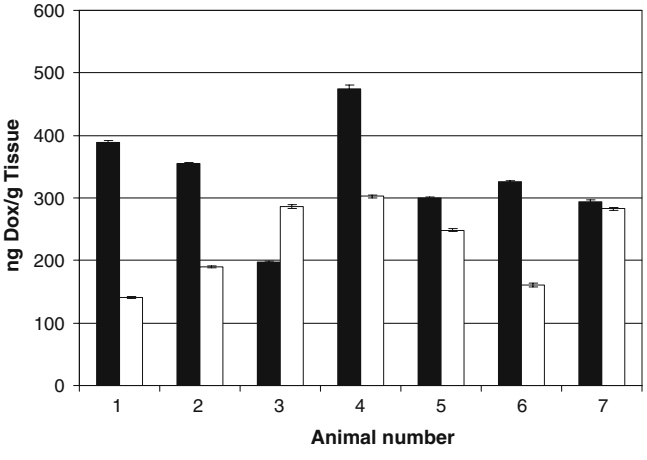

Fig. 3 Doxorubicin concentrations in MDA-MB-231 (*solid bars*) and MDA-MB-231-A1 (*open bars*) tumors from seven female, athymic, BalbC Nu/Nu nude mice, measured by AMS, 2 h following injection with 1 mg/kg body-weight [^{14}C]DOX. *Error bars* represent the coefficient of variation for multiple measurements (up to 7) of the same sample.

For the purpose of demonstrating the utility of AMS, we elected to use a well-described in vitro breast cancer model of drug resistance. The model as previously described [3, 27] uses the multi-drug resistant human breast cancer cell line MDA-MB-231-A1, which has been previously used to demonstrate the accumulation defect once drug resistance has developed to agents such as doxorubicin [3]. The AMS technique showed the difference in anthracycline accumulation over time between wild type and MDR+ breast cancer cells exposed to a fixed concentration of DOX in vitro in a manner consistent with the previously published results [3, 27]. This result was similar to that obtained using HPLC with fluorescence detection. When DOX exposure concentrations were reduced to levels that could not be detected by HPLC, the AMS technique still showed a significantly higher accumulation of anthracycline by the wild type cells at all concentrations, although the difference was less marked at the lower concentrations. Thus, the AMS technique proved to be over five orders of magnitude more sensitive than our HPLC method with fluorescence detection, while still being able to demonstrate the accumulation defect.

When DOX accumulation was examined in vivo, AMS showed a clear difference in accumulation between a low and higher dose of DOX in wild type breast cancer xenografts. This result was in agreement with HPLC data, although concentrations determined by AMS were generally higher. A possible explanation for the higher concentrations determined by AMS is that AMS is unable to distinguish parent drug from metabolites, unlike HPLC. In nude mice bearing both wild type and MDR+ breast cancer xenografts, AMS showed a significantly higher accumulation of anthracycline in the wild type tumors. The demonstration of the accumulation defect by AMS in vivo is significant. With the sensitivity advantages of AMS over other techniques, it may now be possible to determine adequate drug delivery to the target or to identify those patients who may not benefit from standard chemotherapy regimens due to the presence of de novo drug resistance. Ideally this method could be expanded by combining HPLC, which can separate metabolites from parent drug, with AMS, thus giving quantitative information for both parent drug and metabolites [6, 25].

As stated previously, sensitivity in the zeptomole range has been reported using capillary electrophoresis with laser-induced fluorescence to detect metabolites of doxorubicin in subcellular fractions [1, 2]. If this method can be successfully applied to the analysis of biological specimens with the same level of sensitivity as has been demonstrated in vitro, and at least one article suggests that this may be possible [22], it would represent an enormous advancement over current bioanalytical methods for fluorescent agents, similar to the AMS method described here. However, one disadvantage of using AMS compared to CE-LIF methods is that metabolites cannot be distinguished from the parent compound, as mentioned above. Nevertheless, AMS is a novel analytical technique with extreme sensitivity similar to the most sensitive CE-LIF methods available and is another potentially valuable tool for biomedical research. Although a meaningful estimate of the per sample cost of AMS analysis cannot be provided at this time, we anticipate a cost of approximately $100/sample. Additional studies need to be performed to determine the clinical value of using AMS in pharmacodynamic/genomic studies of chemotherapeutics.

Acknowledgements The authors thank Kurt Haack for AMS sample preparation for ^{14}C analysis. This work was performed under the auspices of the US Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48 and supported by a UC Davis Research and Development award from Lawrence Livermore National Laboratory and National Institutes of Health grant 1 P30 CA93373–01 and by the National Center for Research Resources (RR13461).

References

1. Anderson AB, Ciriacks CM, Fuller KM, Arriaga EA (2003) Distribution of zeptomole-abundant doxorubicin metabolites in subcellular fractions by capillary electrophoresis with laser-induced fluorescence detection. Anal Chem 75(1):8–15
2. Anderson AB, Gergen J, Arriaga EA (2002) Detection of doxorubicin and metabolites in cell extracts and in single cells by capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr B 769:97–106
3. Baker WJ, Mäenpää JU, Wurz GT, Koester SK, Seymour RC, Emshoff VD, Wiebe VJ, DeGregorio MW (1993) Toremifene enhances cell cycle block and growth inhibition by vinblastine in multidrug resistant human breast cancer cells. Oncol Res 5(6–7):207–212
4. Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47(3):381–389
5. Citro G, Verdina A, Galati R, Floridi A (1988) Quantitation of adriamycin content by a sensitive immunochemical assay. Anticancer Res 8:549–552
6. Cupid BC, Lightfoot TJ, Russell D, Gant SJ, Turner PC, Dingley KH, Curtis KD, Leveson SH, Turteltaub KW, Garner RC (2004) The formation of AFB(1)-macromolecular adducts in rats and humans at dietary levels of exposure. Food Chem Toxicol 42(4):559–569
7. de Bruijn P, Verweij J, Loos WJ, Kolker HJ, Planting AS, Nooter K, Stoter G, Sparreboom A (2003) Determination of doxorubicin and doxorubicinol in plasma of cancer patients by high-performance liquid chromatography. Anal Biochem 266(2):216–221
8. Gavenda A, Sevcik J, Psotova J, Bednar P, Bartak P, Adamovsky P, Simanek V (2001) Determination of anthracycline antibiotics doxorubicin and daunorubicin by capillary electrophoresis with UV absorption detection. Electrophoresis 22:2782–2785
9. Gottesman M, Fojo T, Bates S (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2(1):48–58
10. Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385–427
11. Hempel G, Haberland S, Schulze-Westhoff P, Mohling N, Blaschke G, Boos J (1997) Determination of idarubicin and idarubicinol in plasma by capillary electrophoresis. J Chromatogr B 698:287–292
12. Lachatre F, Marquet P, Ragot S, Gaulier JM, Cardot P, Dupuy JL (2000) Simultaneous determination of four anthracyclines and three metabolites in human serum by liquid chromatography-electrospray mass spectrometry. J Chromatogr B 738:281–291
13. Lewis JS, Dearling JL, Sosabowski JK, Zweit J, Carnochan P, Kelland LR, Coley HM, Blower PJ (2000) Copper bis(diphosphine) complexes: radiopharmaceuticals for the detection of multidrug resistance in tumours by PET. Eur J Nucl Med 27(6):638–646
14. Maxwell RJ (1993) New techniques in the pharmacokinetic analysis of cancer drugs. III. Nuclear magnetic resonance. Cancer Surv 17:415–423
15. Ognibene TJ, Bench G, Brown TA, Peaslee GF, Vogel JS (2002) A new accelerator mass spectrometry system for ^{14}C-quantification of biochemical samples. Int J Mass Spectrom 218:255–264
16. Ognibene TJ, Bench G, Vogel JS, Peaslee GF, Murov S (2003) A high-throughput method for the conversion of CO_2 obtained from biochemical samples to graphite in septa-sealed vials for quantification of ^{14}C via accelerator mass spectrometry. Anal Chem 75:2192–2196
17. Perez-Ruiz T, Martinez-Lozano C, Sanz A, Bravo E (2001) Simultaneous determination of doxorubicin, danorubicin and iadrubicin by capillary electrophoresis with laser-induced fluorescence detection. Electrophoresis 22:134–138
18. Port R, Hanisch F, Becker M, Bachert P, Zeller J (1999) Local disposition kinetics of floxuridine after intratumoral and subcutaneous injection as monitored by [^{19}F]-nuclear magnetic resonance spectroscopy in vivo. Cancer Chemother Pharmacol 44(1):65–73
19. Reinhoud NJ, Tjaden UR, Irth H, van der Greef J (1992) Bioanalysis of some anthracyclines in human plasma by capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr Biomed Appl 574:327–334
20. Sharma V, Beatty A, Wey S-P, Dahlheimer J, Pica CM, Crankshaw CL, Bass L, Green MA, Welch MJ, Piwnica-Worms D (2000) Novel gallium(III) complexes transported by MDR1 p-glycoprotein: potential PET imaging agents for probing p-glycoprotein-mediated transport activity in vivo. Chem Biol 7(5):335–343
21. Sharma V (2004) Radiopharmaceuticals for assessment of multidrug resistance p-glycoprotein-mediated drug transport activity. Bioconjugate Chem 15(6):1464–1474
22. Simeon N, Chatelut E, Canal P, Nertz M, Couderc F (1999) Anthracycline analysis by capillary electrophoresis: application to the analysis of daunorubicine in Kaposi sarcoma tumor. J Chromatogr A 853:449–454
23. Tilsley DW, Harte RJ, Jones T, Brady F, Luthra SK, Brown G, Price PM (1993) New techniques in the pharmacokinetic analysis of cancer drugs. IV. Positron emission tomography. Cancer Surv 17:425–442
24. Turteltaub KW, Vogel JS (2000) Bioanalytical applications of accelerator mass spectrometry for pharmaceutical research. Curr Pharm Des 6:991–1007

25. Turteltaub KW, Dingley KH, Curtis KD, Malfatti MA, Turesky RJ, Garner RC, Felton JS, Lang NP (1999) Macromolecular adduct formation and metabolism of heterocyclic amines in humans and rodents at low doses. Cancer Lett 143(2):149–155
26. Vogel JS (1992) Rapid production of graphite without contamination for biomedical AMS. Radiocarbon 34:344–350
27. Wiebe V, Koester S, Lindberg M, Emshoff V, Baker J, Wurz G, DeGregorio M (1992) Toremifene and its metabolites enhance doxorubicin accumulation in estrogen receptor negative multidrug resistant human breast cancer cells. Investig New Drugs 10:63–71