Published in final edited form as:

Biomed Chromatogr. 2012 December ; 26(12): 1529–1533. doi:10.1002/bmc.2727.

Confirmation of Drug Delivery after Liver Chemoembolization: Direct Tissue Doxorubicin Measurement by UHPLC-MS-MS

Sigrid Baumgarten¹, Ron C. Gaba², and Richard B. van Breemen^{1,*}

¹Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S. Wood St., Chicago, IL 60612

²Department of Radiology, University of Illinois Medical Center, 1740 W. Taylor St., Chicago, IL 60612

Abstract

Because liver cancer is rarely suitable for surgery, transcatheter arterial chemoembolization (TACE) is used for palliative therapy. In this procedure, an emulsion of doxorubicin in iodized oil is injected directly into liver tumors through a catheter positioned within the artery supplying blood flow to the tumor. At present, there is limited understanding of factors affecting the delivery and dispersion of doxorubicin within treated tumors during TACE. This study addresses the development and application of an ultrahigh pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS-MS) method for rapid confirmation of drug delivery after TACE in a rabbit VX2 liver cancer model. Doxorubicin levels in liver tumors were measured using UHPLC-MS-MS and compared with computed tomography measured levels of iodized oil, a metric used clinically to indicate drug delivery. We found that tissue drug levels determined using UHPLC-MS-MS did not correlate with the regional iodized oil concentration (vehicle) within tumors following TACE, suggesting that chemotherapeutic drugs like doxorubicin spread throughout tumors, and that lack of iodized oil staining in portions of a tumor does not necessarily indicate inadequate therapy during TACE.

Keywords

doxorubicin; UHPLC; tandem mass spectrometry; lipiodol; chemoembolization

Introduction

Liver cancer (hepatocellular carcinoma, HCC) is the fifth most common cancer and the third most common cause of cancer deaths in the world with approximately 550,000 annual deaths, and its incidence is increasing (Parkin *et al.*, 2005; Schutte *et al.*, 2009). The general prognosis for patients with HCC is dismal, with an overall survival rate of 3-5% (Raoul 2008). Therefore, novel therapies are urgently needed for advanced HCC. Surgical resection represents a curative treatment for HCC. However, only a minority (10-15%) of patients with this malignancy are candidates for hepatectomy due to advanced multifocal disease, poor hepatic reserve, or significant extrahepatic tumor burden (Schutte *et al.*, 2009). Hepatic transplantation for HCC is another surgical option but is significantly limited by lack of donor organs.

*Corresponding Author Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S. Wood St., Chicago, IL 60612, Tel: 312-996-9353, FAX: 312-996-7107, breemen@uic.edu.

At present, interventional radiologists treat surgically unresectable HCC with transcatheter arterial chemoembolization (TACE), which represents a recognized locoregional therapy for HCC with proven survival benefit (Llovet *et al.*, 2002; Lo *et al.*, 2002). This procedure may serve as palliative therapy for lifespan prolongation in patients with surgically unresectable HCC (Requarth 2011), may provide a bridge to liver transplantation in patients within the Milan criteria (Dhanasekaran *et al.*, 2010; Lewandowski *et al.*, 2009), and may facilitate disease down-staging in patients beyond transplant criteria (Lewandowski *et al.*, 2009; Chapman *et al.*, 2008). In this procedure, targeted therapy consisting of cytotoxic chemotherapeutic drugs is delivered directly to tumors via the arteries that supply them. The chemotherapy is administered as an emulsified mixture with an iodized poppy seed oil derivative (Gaba 2011; Satake *et al.*, 2008). The iodized oil acts as a carrier for chemotherapy drugs, facilitates intracellular transit of these medications (Owu *et al.*, 2004), and serves as an embolic agent that occludes tumor blood vessels and reduces tumor perfusion, increasing contact time between tumor cells and cytotoxic drugs (Ramsey *et al.*, 2002). The degree of iodized oil accumulation within tumors has also been used to estimate the technical success and therapeutic efficacy of chemoembolization treatment (Taguchi *et al.*, 2009; Fujita *et al.*, 2008).

During imaging, the regional distribution and quantity of iodized oil within tumors are thought to be associated with local intra-tumoral drug concentration and are utilized as a metric for determining successful local chemotherapeutic agent deposition during TACE (Taguchi *et al.*, 2009). While studies performed to date have independently assessed iodized oil (Kozutsumi *et al.*, 2000) or chemotherapy (Wang *et al.*, 2009) levels within tumor tissue following chemoembolization, direct correlation of intra-tumoral iodized oil content and local chemotherapy concentration has not been performed. The determination of such a correlation would help quantify the actual amount of drug delivered, which in turn might help elucidate the relative contribution of cytotoxic versus hypoxic cellular necrosis mechanisms in chemoembolization. Improvement of drug delivery has the potential to further enhance the benefits of chemoembolization and increase patient lifespan.

In this study, rabbits with liver tumors were administered the chemotherapeutic agent doxorubicin in an emulsion with the iodinated oil, Lipiodol, using TACE. Doxorubicin is an anthracycline that is closely related to the natural product daunorubicin (Figure 1) (Licata *et al.*, 2000) and has become a standard chemotherapeutic agent for TACE (Weiss 1992). The regional distribution of doxorubicin within tumors was compared with Lipiodol levels. This investigation was designed to validate the presumed association between regional intra-tumoral iodine concentration (the current clinical gold standard to indicate the success of chemotherapeutic drug delivery) and local tissue chemotherapeutic drug levels. Although a liquid chromatography–tandem mass spectrometry (LC–MS–MS) method has been reported for the quantification of doxorubicin in tissue samples (Arnold *et al.*, 2004), we developed a faster assay based on ultrahigh pressure LC–MS–MS (UHPLC–MS–MS) to quantify the regional distribution of doxorubicin in liver tissue following TACE. Then, these UHPLC–MS–MS results for doxorubicin were correlated with computed tomography (CT) imaging of the regional tissue concentration of iodine.

Experimental

Chemicals and reagents

Doxorubicin and daunorubicin were purchased from McKesson (San Francisco, CA) and Sigma-Aldrich (St. Louis MO), respectively, and Lipiodol was purchased from Guerbet (Villepinte, France). All organic solvents were HPLC-grade or better and were purchased from ThermoFisher (Pittsburg, PA). Purified water was prepared using a Millipore Milli-Q purification system (Millipore, Billerica, MA).

Sample preparation

After study approval by our hospital's Animal Care and Use Committee, VX2 cell line development, propagation, and implantation into the livers of New Zealand white rabbits were performed according to the methodology of Virmani *et al.* (2008). VX2 carcinoma is an anaplastic squamous cell carcinoma derived from a virus-induced papilloma in rabbits, and represents a widely used liver cancer model (Ko *et al.* 2001). Two tumors (mean 1.9 cm diameter) were developed in each left hepatic lobe of two male New Zealand white rabbits (each 3 kg). TACE was then carried out by injection of an emulsion of doxorubicin hydrochloride (2 mg) and Lipiodol (1 mL) into each of 4 VX2 liver tumors. Rabbits were euthanized immediately upon completion of chemoembolization, and CT scanning was carried out within 30 min of euthanasia (see details below). The four liver tumors were removed from the rabbits within 30 min after completion of the CT scanning, and the central tumor slice was cut into 5 pieces each, which were homogenized in phosphate buffer (0.05 M, pH 7.4) to produce a homogenate containing 0.2 g tissue per mL. Homogenized tissue was extracted with four volumes of ice-cold acetonitrile and then centrifuged at 5,000g for 10 min. Each supernatant was then removed, and daunorubicin was added as an internal standard at a concentration of 100 ng/mL. Each extract was evaporated to dryness and reconstituted in UHPLC mobile phase immediately before analysis.

UHPLC-MS-MS

Extracts were analyzed using UHPLC-MS-MS on a Shimadzu (Kyoto, Japan) Nexera UHPLC system interfaced to a Shimadzu LCMS-8030 triple quadrupole mass spectrometer. UHPLC separations were carried out using a Shimadzu Shim-Pack ODS column (2.0 × 50 mm, 1.6 μm) with a mobile phase consisting of a 1.5 min gradient from 20:80 to 50:50 (v/v) acetonitrile/5 mM aqueous ammonium acetate (pH 3.5) at a flow rate of 0.5 mL/min. The autosampler was maintained at 15 °C, and the injection volume was 5 μL. Aqueous ammonium acetate adjusted to pH 3.5 using glacial acetic acid was used instead of formic acid to achieve optimum chromatographic separation and sensitivity for the analysis of doxorubicin as reported by Arnold *et al.* (2004).

Doxorubicin and the internal standard daunorubicin were ionized using positive ion electrospray with a capillary temperature of 250 °C and a spray voltage of 4.5 kV. During collision-induced dissociation, fragmentation of doxorubicin and daunorubicin was carried out at -15 eV and -30 eV, respectively. Quantitative analysis was carried out using the SRM mass transitions of m/z 544 to m/z 397 and m/z 528 to m/z 321 for doxorubicin and daunorubicin as quantifiers, respectively. The SRM transitions of m/z 544 to m/z 361 and m/z 528 to m/z 363 were monitored as qualifiers for doxorubicin and daunorubicin, respectively. The SRM dwell time was 20 ms per ion, and the pause time between SRM measurements was 1 ms.

Standard curve

Standard curves were prepared using blank tissues as the matrix. After tissue homogenization, doxorubicin was added immediately before extraction with ice-cold acetonitrile as described above. The range of doxorubicin standard concentrations was 0.1 ng/mL to 10,000 ng/mL, and standards were analyzed using UHPLC-MS-MS.

Computed tomography (CT) measurement of tumor Lipiodol content

CT imaging, obtained within 30 min of TACE, was performed using a BrightSpeed 16 slice scanner (GE Healthcare, United Kingdom) with helical acquisition and mA = 300-400, kV = 120, pitch = 1.375:1, and 0.625 mm acquisition slice thickness. CT calculation of tissue iodine content was performed according to the methodology of Taguchi *et al.* (2009) Axial CT

slice positions at the center of each treated tumor were selected and divided into five regions-of-interest corresponding to the tumor sections used for tissue analysis.

Results and discussion

During UHPLC-MS-MS, doxorubicin eluted at 0.89 min and daunorubicin eluted at approximately 1.1 min (Figure 2). The total time between injections was 1.5 min using UHPLC with a gradient solvent system compared with 5.0 min between injections for the isocratic HPLC method of Arnold *et al.* (2004). The UHPLC-MS-MS standard curve was linear from 0.5 to 10,000 ng/mL ($y=14.9x + 957.9$) with a coefficient of determination, R^2 , of 0.9993. The lower limit of quantitation for doxorubicin was 0.5 nM. The ratios of the SRM quantifier and qualifier peak areas were 3.58 ± 0.21 (STD DEV) and 3.60 ± 0.19 for doxorubicin and daunorubicin, respectively. The use of both quantifier and qualifier SRM transitions in the method enhanced the confidence that the analyte and internal standard were identified correctly, and that there was no matrix interference during the analysis.

This UHPLC-MS-MS method was similar to the LC-MS-MS method of Arnold, *et al.* (2004) with respect to accuracy and precision as well as analyte recovery and stability. For example, at a nominal concentration of 5000 ng/ml (9.2 μ M), recovery was >82%, and interday assay precision was 6.2% compared with Arnold *et al.* (2004) who reported >84% recovery and an interassay precision of 7.1% for a nominal concentration of 2.5 μ M doxorubicin. Whereas Arnold *et al.* (2004) had used the SRM transition of m/z 544 to m/z 361 for the measurement of doxorubicin, we used this transition as the qualifier and added the SRM transition of m/z 544 to m/z 397 as the quantifier. As reported previously (Monneret and Sellier 1986), the initial site of protonation of anthracycline antibiotics like doxorubicin occurs primarily on the oxygen of the glycosidic bond, resulting in abundant fragmentation at that position with the oxygen atom remaining on the sugar moiety to form the doxorubicin product ion of m/z 397.

Doxorubicin concentrations in 20 liver tumor and tissue samples were determined using the new UHPLC-MS-MS method and are summarized in Table 1. Doxorubicin was detected in all samples, and levels were approximately the same in both the peripheral tissue (7.5 ± 3.0 μ g/mL, $n = 16$) and core tumor (6.9 ± 3.1 μ g/mL, $n = 4$) sections ($P = 0.721$). In contrast, when the intra-tumoral distribution of radiopaque Lipiodol was determined non-invasively using post-TACE CT scanning (Figure 3 and Table 1), the iodine content was significantly greater along the peripheral, viable portions of treated tumors (498 ± 201 mg/mL, $n = 16$) as compared with the central core (164 ± 150 mg/mL, $n = 4$) ($P = 0.006$).

Tissue drug levels did not correlate with the calculated regional iodized oil concentration within tumors following TACE, indicating that the degree of intra-tumoral Lipiodol deposition is a poor quantitative predictor of local doxorubicin concentration after liver tumor chemoembolization. These results oppose the hypothesis that Lipiodol is an accurate imaging marker for doxorubicin drug delivery, challenge the fidelity of this agent as a dependable imaging marker for doxorubicin drug delivery, and highlight the need to identify a more accurate non-invasive quantitative indicator of drug delivery during conventional TACE therapy.

Conclusions

Not applied previously in studies of liver chemoembolization, this investigation addressed the use of UHPLC-MS-MS for validation of drug delivery after transcatheter arterial chemoembolization in rabbit VX2 tumor HCC. Although previous studies have used LC-MS-MS to quantify concentrations of chemotherapy drugs in serum (Arnold *et al.*, 2004; Sottani *et al.*, 2009) and liver tissue (Arnold *et al.*, 2004), we extended this approach to

include the faster technique of UHPLC-MS-MS for the measurement of doxorubicin uptake in tissue after chemoembolization. In the UHPLC-MS-MS chromatogram shown in Figure 2, the width of each peak (doxorubicin and daunorubicin) was only 3 s, and both peaks were resolved completely while eluting within approximately 1 min. For comparison, a recent LC-MS-MS method (Arnold *et al.*, 2004) was more than 3-fold slower and did not completely resolve the doxorubicin and daunorubicin peaks. Using the new UHPLC-MS-MS method to evaluate the tissue distribution of doxorubicin in liver tissue during TACE, we found no correlation between tissue iodine (Lipiodol) and doxorubicin levels in treated tumor tissue. This indicates that Lipiodol deposition in the liver is a poor quantitative predictor of local doxorubicin concentration after liver tumor chemoembolization in the rabbit VX2 model

Acknowledgments

We thank the Shimadzu Corporation for providing the UHPLC-MS-MS system used during this investigation.

References

- Arnold RD, Slack JE, Straubinger RM. Quantification of doxorubicin and metabolites in rat plasma and small volume tissue samples by liquid chromatography/electrospray tandem mass spectroscopy. *Journal of Chromatography B*. 2004; 808:141–152.
- Chapman WC, Majella Doyle MB, Stuart JE, Vachharajani N, Crippin JS, Anderson CD, Lowell JA, Shenoy S, Darcy MD, Brown DB. Outcomes of neoadjuvant transarterial chemoembolization to downstage hepatocellular carcinoma before liver transplantation. *Annals of Surgery*. 2008; 248:617–625. [PubMed: 18936575]
- Dhanasekaran R, Khanna V, Kooby DA, Spivey JR, Parekh S, Knechtle SJ, Carew JD, Kauh JS, Kim HS. The effectiveness of locoregional therapies versus supportive care in maintaining survival within the Milan criteria in patients with hepatocellular carcinoma. *Journal of Vascular and Interventional Radiology*. 2010; 21:1197–1204. [PubMed: 20656222]
- Fujita T, Ito K, Tanabe M, Yamatogi S, Sasai H, Matsunaga N. Iodized oil accumulation in hypervascular hepatocellular carcinoma after transcatheter arterial chemoembolization: comparison of imaging findings with CT during hepatic arteriography. *Journal of Vascular and Interventional Radiology*. 2008; 19:333–341. [PubMed: 18295691]
- Gaba RC. Abstract No. 283: A survey of chemoembolization practice patterns and technical methodologies among interventional radiologists. *Journal of Vascular and Interventional Radiology*. 2011; 22:S119–S120.
- Ko YH, Pedersen PL, Geschwind JF. Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase. *Cancer Letters*. 2001; 173:83–91. [PubMed: 11578813]
- Kozutsumi D, Ohshiba Y, Sugimoto T, Tsuboi H, Kawashima A, Kochiya M, Adachi M, Takami M. A new assay for lipiodol in a tumor using a combination of *m*-chloroperbenzoic acid-mediated oxidation and the iodo-starch reaction. *Cancer Letters*. 2000; 158:93–98. [PubMed: 10940514]
- Lewandowski RJ, Kulik LM, Riaz A, Senthilnathan S, Mulcahy MF, Ryu RK, Ibrahim SM, Sato KT, Baker T, Miller FH, Omary R, Abecassis M, Salem R. A comparative analysis of transarterial downstaging for hepatocellular carcinoma: chemoembolization versus radioembolization. *American Journal of Transplantation*. 2009; 9:1920–1928. [PubMed: 19552767]
- Licata S, Saponiero A, Mordente A, Minotti G. Doxorubicin metabolism and toxicity in human myocardium: role of cytoplasmic deglycosidation and carbonyl reduction. *Chemical Research in Toxicology*. 2000; 13:414–420. [PubMed: 10813659]
- Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Solá R, Rodés J, Bruix J. Barcelona Clinic Liver Cancer Group. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet*. 2002; 359:1734–1739. [PubMed: 12049862]

- Lo CM, Ngan H, Tso WK, Liu CL, Lam CM, Poon RT, Fan ST, Wong J. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology*. 2002; 35:1164–1171. [PubMed: 11981766]
- Monneret C, Sellier N. Desorption chemical ionization mass spectrometry of anthracyclines and of trisaccharides related to aclacinomycin A and marcellomycin. *Biomedical & Environmental Mass Spectrometry*. 1986; 13:319–326. [PubMed: 2943345]
- Owu E, Al-Mufti R, Winslet M. Uptake of lipiodol-cytotoxics conjugates by hepatocellular carcinoma cells. *Journal of Pediatric Surgery*. 2004; 39:203–206. [PubMed: 14966741]
- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA: a Cancer Journal for Clinicians*. 2005; 55:74. [PubMed: 15761078]
- Ramsey DE, Kernagis LY, Soulen MC, Geschwind JH. Chemoembolization of hepatocellular carcinoma. *Journal of Vascular and Interventional Radiology*. 2002; 13:S211–S221. [PubMed: 12354839]
- Raoul JL. Natural history of hepatocellular carcinoma and current treatment options. *Seminars in Nuclear Medicine*. 2008; 38:S13–S18. [PubMed: 18243838]
- Requarth J. Image-guided palliative care procedures. *The Surgical Clinics of North America*. 2011; 91:367–402. [PubMed: 21419259]
- Satake M, Uchida H, Arai Y, Anai H, Sakaguchi H, Nagata T, Yamane T, Kichikawa K, Osaki Y, Okazaki M, Higashihara H, Nakamura H, Osuga K, Nakao N, Hirota S. Transcatheter arterial chemoembolization (TACE) with lipiodol to treat hepatocellular carcinoma: survey results from the TACE study group of Japan. *Cardiovascular and Interventional Radiology*. 2008; 31:756–761. [PubMed: 18389187]
- Schutte K, Bornschein J, Malfertheiner P. Hepatocellular carcinoma--epidemiological trends and risk factors. *Digestive Diseases (Basel, Switzerland)*. 2009; 27:80–92.
- Sottani C, Leoni E, Porro B, Montagna B, Amatu A, Sottotetti F, Quaretti P, Poggi G, Minoia C. Validation of an LC-MS/MS method for the determination of epirubicin in human serum of patients undergoing drug eluting microsphere-transarterial chemoembolization (DEM-TACE). *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences*. 2009; 877:3543–3548.
- Taguchi K, Funama Y, Zhang M, Fishman EK, Geschwind JF. Quantitative measurement of iodine concentration in the liver using abdominal C-arm computed tomography. *Academic Radiology*. 2009; 16:200–208. [PubMed: 19124106]
- Virmani S, Harris KR, Szolc-Kowalska B, Paunesku T, Woloschak GE, Lee FT, Lewandowski RJ, Sato KT, Ryu RK, Salem R, Larson AC, Omary RA. Comparison of two different methods for inoculating VX2 tumors in rabbit livers and hind limbs. *Journal of Vascular and Interventional Radiology*. 2008; 19:931–936. [PubMed: 18503910]
- Wang Y, Hong J, Cressman EN, Arriaga EA. Direct sampling from human liver tissue cross sections for electrophoretic analysis of doxorubicin. *Analytical Chemistry*. 2009; 81:3321–3328. [PubMed: 19323500]
- Weiss RB. The anthracyclines: will we ever find a better doxorubicin? *Seminars in Oncology*. 1992; 19:670–686. [PubMed: 1462166]

Abbreviations

TACE	transcatheter arterial chemoembolization
HCC	hepatocellular carcinoma
UHPLC-MS-MS	ultra high pressure liquid chromatography-tandem mass spectrometry

Figure 1.
Chemical structures of doxorubicin and the internal standard daunorubicin.

Figure 2. UHPLC-MS-MS chromatograms of doxorubicin and daunorubicin (100 ng/mL each) extracted from rabbit liver obtained using positive ion electrospray with collision-induced dissociation and selected reaction monitoring (SRM).

Figure 3.

Non-contrast rabbit CT scan following conventional TACE of VX2 liver tumor showing differential distribution of iodized oil (Lipiodol) within tumor. Image enlargement shows regions used for CT iodine calculation as well as the sections that were isolated for measurement of doxorubicin. (AL = anterior lateral tumor segment, PL = posterior lateral tumor segment, PM = posterior medial tumor segment, AM = anterior medial tumor segment, C = tumor core)

Table 1

Doxorubicin and iodine levels of rabbit liver tumors following TACE using an emulsion of doxorubicin in the iodinated oil Lipiodol.

Rabbit	Tumor segment	Tissue volume (mL)	Lipiodol dose (mL)	Doxorubicin (mg)	Iodine content (µg/mL)	Doxorubicin level (µg/mL)
1	1	AM [/]	1	2	692	7.9
		PM			446	9.0
		PL			481	9.5
		AL			530	5.2
		C			155	11.0
	2	AM			573	5.2
		PM			899	8.2
		PL			573	6.6
		AL			448	6.1
		C			378	5.7
2	1	AM	1	2	632	4.7
		PM			333	11.3
		PL			381	7.3
		AL			764	15.9
		C			49	2.7
	2	AM			426	6.3
		PM			348	7.8
		PL			433	3.9
		AL			18	5.6
		C			75	11.5

[/]AM = anterior medial tumor segment; PM = posterior medial tumor segment; PL = posterior lateral tumor segment; AL = anterior lateral tumor segment; C = tumor core