Quantification of [^{3}H]docetaxel in feces and urine: development and validation of a combustion method

Frederike K. Engels[a], Dirk Buijs[b], Walter J. Loos[a], Jaap Verweij[a], Willem H. Bakker[b] and Eric P. Krenning[b]

Most radiolabeled biological samples require extensive sample preparation to reduce quenching interference before quantification of radioactivity is possible. Clearly, a more rapid and simple method ensuring a constant count rate and optimal counting efficiency has important advantages. We report on the development and analytical method validation of a rapid and simple combustion method to quantify [^{3}H]docetaxel excreted in human feces and urine. A 3-day validation procedure was performed; quality control (QC) samples, prepared in blank feces and urine, were combusted 5 times and aliquots of the produced tritiated combustion water were counted in a liquid scintillation counter. The validation runs demonstrated adequate precision (below 7.6%) across all QC levels. Sensitivity at the lowest QC level was excellent and recovery of radioactivity constant (ranging from 85 to 91.8%). Clinical applicability of the method was tested in a cancer patient receiving docetaxel and a tracer amount of [^{3}H]docetaxel; during the first 72 h after [^{3}H]docetaxel infusion, 60% of total radioactivity was excreted in the collected feces and urine, which is within the expected range. Combustion of tritiated feces and urine samples is a simple, rapid, sensitive, precise and reproducible method with high recovery. It can be applied to quantify [^{3}H]docetaxel excretion after i.v. administration. *Anti-Cancer Drugs* 17:63–67 © 2006 Lippincott Williams & Wilkins.

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Departments of [a]Medical Oncology and [b]Nuclear Medicine, Erasmus MC – Daniel den Hoed Cancer Center, Rotterdam, The Netherlands.

Correspondence to F.K. Engels, Department of Medical Oncology, Erasmus MC – Daniel den Hoed Cancer Center, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands.
Tel: +31 10 4391 937; fax: +31 10 4391 053;
e-mail: f.engels@erasmusmc.nl

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Introduction

An important reason to administer a non-toxic tracer amount of radiolabeled drug to a patient is to quantify the drug in biological samples (e.g. plasma, feces, urine, ascites) at detection levels below the lower limit of quantification for routinely used analytical techniques (e.g. HPLC). Radiochemicals used in (clinical) research include ^{3}H- or ^{14}C-radiolabeled compounds. Liquid scintillation counting (LSC) is the most efficient method to quantify these low-energy β-emitters. Most radiolabeled biological samples however, cannot be directly subjected to LSC and first require a sample preparation procedure, e.g. to reduce color or chemical quenching interference. Indeed with LSC the detection and quantification limit is affected by sample size, sample preparation method and counting efficiency. Ideally, the sample size is as large as possible, and pre-treatment is simple, rapid and results in a clear, homogeneous and stable mixture of radioactive sample and appropriate liquid scintillation cocktail, thus allowing for a constant count rate and optimal counting efficiency. This ultimately gives the most accurate and reproducible counting results. Various sample preparation procedures to solubilize a biological matrix are applied and include acid or alkaline digestion. Indeed, in preclinical mass balance studies with ^{3}H-radiolabeled paclitaxel and docetaxel, feces sample pre-treatment involved initial homogenization with 4% bovine serum albumin in water, followed by a 1.5-h incubation with a (tissue) solubilizer (Soluene) and a 2-h incubation with 2-propanol [1,2]. Finally, 30% hydrogen peroxide, necessary to bleach the samples, was added before mixing with a compatible liquid scintillation cocktail (Hionic-Fluor). Combustion of biological samples is an alternative sample preparation method [3–6] and has several practical advantages. Combustion of a ^{3}H-radiolabeled sample produces a colorless amount of tritiated (combustion) water, which can be counted immediately after homogeneously mixing with liquid scintillation cocktail. It is a rapid method, quenching interference is avoided and sample size can be increased compared to acid or alkaline digestion, as such positively influencing the detection and quantification limit.

The anti-cancer drug docetaxel (Taxotere) is approved for the treatment of patients with locally advanced or metastatic breast cancer, non-small cell lung cancer and androgen-independent metastatic prostate cancer (www.taxotere.com). After i.v. administration the drug is extensively and predominantly metabolized by the hepatic and intestinal cytochrome P450 isozyme 3A

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(CYP3A) [7,8]. Indeed, the major metabolites of docetaxel and less than 10% of the parent drug are excreted into the feces, whereas total urinary excretion is less than 10% [9,10]. Preclinical metabolism and excretion studies with ^{14}C-radiolabeled docetaxel have demonstrated that the elimination of radioactivity in mice, dogs and rats was rapid, and almost complete (85–95%) within 7 days after administration [11,12]. Clinical studies assessing excretion and metabolism of ^{14}C-radiolabeled docetaxel are limited [13]. In patients ($n = 3$) approximately 80% of total administered radioactivity was excreted in the 7-day feces, with the majority of excretion occurring during the first 48 h and only 5% of total radioactivity was retrieved in urine during the first 6 h after administration. The catalytic activity of the isozyme CYP3A is readily modulated by inducing or inhibiting compounds such as drugs, dietary supplements, and complementary and alternative medicines [14,15]. Although docetaxel is subject to a host of CYP3A-mediated interactions that can affect treatment outcome, data on potential interactions are lacking. We therefore recently conducted a study to evaluate the effect of co-administration of the potent CYP3A inhibitor ketoconazole (Nizoral) on the pharmacokinetics of docetaxel in cancer patients [14]. A secondary objective of this project was to assess the influence of ketoconazole on the excretion profile and mass balance of docetaxel and its metabolites in feces and urine samples using a radiolabeled tracer amount of [^{3}H]docetaxel. In the context of this secondary objective we now report on the development and analytical method validation of a combustion method to quantify [^{3}H]docetaxel excreted in feces and urine samples according to international guidelines for bioanalytical method validation [16].

Materials and methods

Chemicals and solutions

Tritiated docetaxel ([^{3}H]docetaxel; 1.0 mCi/ml in ethanol, specific activity 7.2 Ci/mmol) was manufactured by Moravek Biochemicals (Brea, California, USA) and 5-ml aliquots (Working Stock Solution) of a 200-fold dilution in ethanol absolute (above 99.9%; Merck, Darmstadt, Germany) were stored at −80°C. Purity of the [^{3}H]docetaxel was analyzed at two intervals (directly after purchase and prior to patient administration) by a reversed-phase HPLC method with UV detection at 230 nm [17]. Briefly, to 10 µl docetaxel stock solution (1.0 mg/ml) prepared by dissolving the appropriate amount of drug (purity 99.3%; Aventis Pharma, Hoevelaken, The Netherlands) in DMSO (purity 99.9%; Sigma, St Louis, Missouri, USA), 100 µl of [^{3}H]docetaxel working stock solution and 390 µl of purified water was added and mixed; a 100-µl aliquot of this solution was injected on the HPLC system and the eluted mobile phase collected in polyethylene vials every 30 s up to 15 min after injection; 150 µl of each fraction of collected eluted mobile phase was mixed with 2 ml liquid scintillation cocktail (Emulsifier Scintillator 299; Packard Bioscience, Groningen, The Netherlands). The collected fractions and a corresponding blank were counted in a liquid scintillator counter (Wallac 1409; Perkin-Elmer, Boston, Massachusetts, USA). To assess purity of the [^{3}H]docetaxel lot, the time course (i.e. elution profile) of the recovered radioactivity was compared with the chromatographic profile of (unlabeled) docetaxel. Feces and urine were obtained from healthy volunteers. Feces was homogenized in 3 volumes (1:3 w/v) of PBS solution (Oxoid, Basingstoke, UK) using an Ultra-TurraxT25 (Janke & Kunkel, IKA Labortechnik, Staufen, Germany) homogenizer (Kinematika, Lucerne, Switzerland) and stored at −20°C. Pooled urine samples did not undergo any processing and were immediately stored at −20°C. Water purified and deionized by the Milli-Q UF Plus system (Millipore, Bedford, Massachusetts, USA) was used in all aqueous solutions.

Quality control (QC) sample preparation

Three levels of QC samples (QC-low, QC-medium and QC-high) were prepared in homogenized blank feces and two levels in pooled blank urine (QC-low and QC-high). For this purpose a 1:10 and a 1:100 dilutions (in PBS) of [^{3}H]docetaxel working stock solution were prepared. Weighted aliquots of 50 g of blank homogenized feces was spiked with 800 or 300 µl of the 1:10 dilution to prepare the QC-high and QC-medium samples, respectively; 50 µl of the same dilution was used to spike 50 ml of blank urine (QC-high). Similarly weighted aliquots of 50 g of homogenized blank feces and 50 ml of blank urine were both spiked with 50 µl 1:100 dilution to produce QC-low samples. The chosen levels of radioactivity were based on preliminary data of measurements in patient samples at time points when maximum and minimum excretion was expected. All QC samples were stored at −20°C until analysis. For recovery calculation the nominal level of radioactivity in the amounts of [^{3}H]docetaxel used for spiking was measured in duplicate by LSC.

Measurement of radioactivity levels

The radionuclide fraction of ^{3}H was obtained after complete combustion of an aliquot of each QC sample in a closed-type combustion flask (Parr; flask volume 300 ml), thus reducing the sample to ash and yielding tritiated (combustion) water (^3H_2O). Briefly, after accurately weighing the combustion flask a known amount (approximately 1.0–1.5 g) of sample was placed in the sample basket (1.5 cm high and 3 cm diameter) with a copper ignition wire connected to it. Additionally, for urine samples a piece of filter paper was added to absorb the sample and prevent it from spilling out of the basket. The combustion flask was then hermetically closed and filled with 25 atm oxygen using an oxygen cylinder with a pressure regulator. Combustion was started by applying an electric current (12 V) to the ignition wire. After combustion, the flask was cooled in an ice-water bath for approximately 5 min. The combustion

flask was then weighed again to determine the total produced amount of tritiated combustion water. Finally, to an exact (i.e. known) amount (approximately 2 ml) of tritiated combustion water 18 ml liquid scintillation cocktail (Emulsifier Scintillator 299) was added and the solution manually mixed in glass counting vials (Econo glass vial, 20 ml; Packard Bioscience) until homogeneous. Samples were counted in a liquid scintillator counter (Wallac 1409; Perkin-Elmer). Counting time was set at 1 h per sample, and in each run a corresponding combustion blank and a ^{3}H standard were included to correct for background radioactivity and to check stability of the counting instrument, respectively. Using the known amounts of combusted sample and produced and counted tritiated combustion water, the total amount of radioactivity in the QC samples could be calculated.

Validation of precision and recovery

The validation procedure was performed on three separate days (i.e. runs) and each feces or urine QC sample was processed (i.e. combusted) 5- or 6 times, respectively. The precision of the assay was assessed by between-run precision (reproducibility) and within-run precision (repeatability). Estimates of the between-run precision and within-run precision were obtained for each (radioactivity) level by one-way analysis of variance (ANOVA) using the run day as classification variable. The between-groups mean square (MS_{betw}), the within-groups mean square (MS_{with}) and the grand mean (GM) of the observed radioactivity levels across run days were calculated in the SPSS for Windows, version 9.0 (SPSS; Chicago, Illinois, USA). The between-run precision (BRP) was defined as: $BRP\ (\%) = \{[(MS_{betw} - MS_{with}/n]^{0.5}/GM\} \times 100\%$, where n represents the number of replicate measurements within each validation run. The within-run precision (WRP) was calculated as: $WRP\ (\%) = \{(MS_{with})^{0.5}/GM\} \times 100\%$. In cases where the MS_{with} is greater than the MS_{betw}, the resulting variance estimate is negative, implying that no additional variance was observed as a result of performing the assay in different runs. The average recovery (REC) for each QC sample was calculated as: $REC = (GM/\text{nominal radioactivity level}) \times 100\%$. The bioanalytical method validation is accepted when the WRP and BRP at each level of QC samples are 15% or less and at least 80% of the QC samples at each level have an absolute deviation of 15% or less of their nominal concentration [16].

Lower limit of quantification

In general, for a concentration (i.e. level of radioactivity) to be acceptable as the lowest level of quantification the percentage of deviation from the nominal value of at least 80% of the samples assayed should be 20% or less, with a resulting WRP of 20% or less [16]. Although no samples were spiked with levels of radioactivity lower than the QC-low nominal values, calculation of the statistical error [standard deviation (SD) and coefficient of variation (CV)] for the accumulated counts for the QC-low samples gives an impression of the level of quantification (i.e. sensitivity), which can be achieved. The SD in the net count rate after correcting the gross count rate (R_a) for the blank (R_b) was calculated using the following error formula: $SD = \sqrt{(R_a/t_a + R_b/t_b)}$, where the counting time for both sample and blank (t_a and t_b) was 60 min. The CV (%) was calculated as: $CV\ (\%) = \sqrt{\{1/(R_a t_a) + 1/(R_b t_b)\}} \times 100\%$.

Clinical applicability

Complete 24-h urine and feces were collected up to 72 h after a 1-h i.v. infusion of 210 mg docetaxel (dose 100 mg/m^2) and a tracer amount (approximately 50 μCi) of [^{3}H]docetaxel to a cancer patient participating in the previously mentioned study [14]. Samples were stored, processed and analyzed (in duplicate) as described for the QC samples. The observed radioactivity levels in the samples were used to calculate the total amount of radioactivity recovered in each collected feces and urine sample, and the percentage of recovered radioactivity (i.e. relative to the amount which was added to the docetaxel infusion) was determined.

Potential loss of added [^{3}H]docetaxel due to adhesion to the infusion bag (NaCl 0.9%, 250 ml, Viaflex; Baxter, Utrecht, The Netherlands), i.v. administration set (Graseby, Watford, UK; ref. 591.078J) or connect set (Codan, Lensahn, Germany; ref. 76.4440) was assessed. For this purpose the clinical practice of infusion was simulated using two docetaxel infusions (20 and 160 mg docetaxel) which both contained the same amount of [^{3}H]docetaxel used in the clinical trial [14]. After the simulated infusion, duplicate specimens of the infusion bags and lines were combusted, and the combustion water distillated to produce a clear sample, mixed with liquid scintillation cocktail and followed by LSC to determine the level of radioactivity.

Results

Method validation

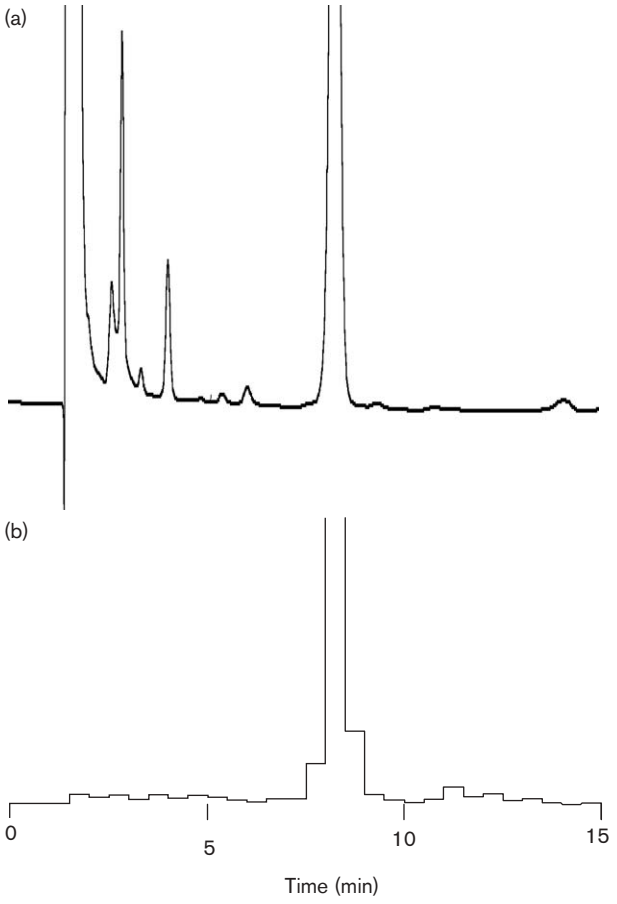
No signs of degradation or decreased purity of the lot of [^{3}H]docetaxel used for validation of the combustion

Table 1 Within-run precision, between-run precision and recovery of [^{3}H]-docetaxel

Quality control sample	Nominal (dpm/g)	Observed (dpm/g)	WRP (%)	BRP (%)	*n*	REC (%)
Feces						
QC-low	190	170	5.2	#	5	89.2 (4.4)
QC-medium	13722	12425	1.8	#	5	90.5 (1.5)
QC-high	35344	32457	2.0	1.5	5	90.5 (1.5)
Urine						
QC-low	186	158	7.6	#	6	85.0 (6.4)
QC-high	2147	1967	2.2	#	6	91.6 (2.0)

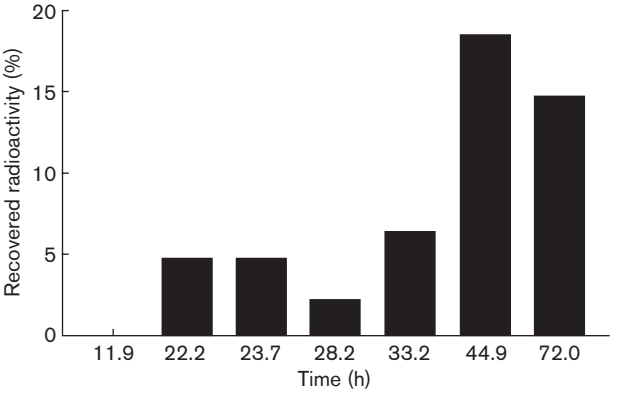
Abbreviations: WRP, within-run precision; BRP, between-run precision; *n* number of replicate observations within each validation run; REC, mean recovery (standard deviation) for all observations. #, no additional variation was observed by performing the assay in different runs.

Fig. 1



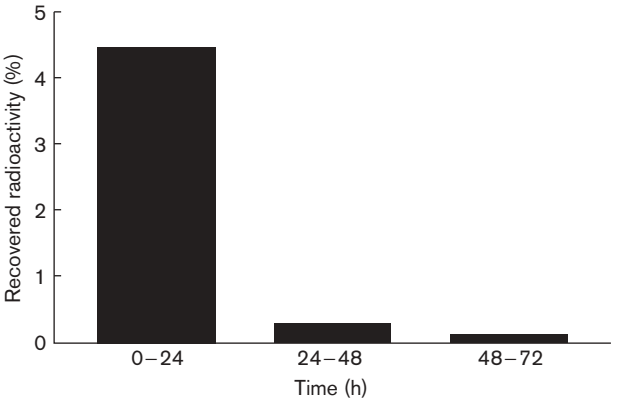
Chromatographic tracing with UV detection (a) and off-line liquid scintillation counting (b) after injection of docetaxel and an added tracer amount of [^{3}H]docetaxel.

Fig. 2



Recovery radioactivity (%) in feces samples collected up to 72 h after i.v. administration of 210 mg docetaxel and an added tracer amount of [^{3}H]docetaxel.

Fig. 3



Recovery radioactivity (%) in 24-h urine samples up to 72 h after i.v. administration of 210 mg docetaxel and an added tracer amount of [^{3}H]docetaxel.

method and throughout the clinical trial was observed, even after a period of prolonged storage at −80°C. More than 90% of the radioactivity recovered in the collected fractions of the eluted mobile phase after chromatographic analysis of [^{3}H]docetaxel was detected at the retention time of docetaxel (Fig. 1).

At each of the three or two, respectively, QC levels of [^{3}H]docetaxel in homogenized feces and urine the within-run precision and between-run precision was below 7.6% (Table 1). The ratio of the volumes of [^{3}H]docetaxel 1:10 dilution used to spike the feces QC samples (QC-medium 300 µl:QC-high 800 µl = 1.0:2.7) could also be calculated for the observed levels (grand mean) in the corresponding samples (QC-medium:QC-high = 1.0:2.6), indicating linearity in the counting results. Similar results for linearity were seen in the urine samples. Furthermore, the ratio of the observed (*GM*) levels in the QC-low samples for the two matrices both spiked with 50 µl 1:100 dilution were as expected (QC-low urine:QC-low feces = 1.0:1.1). These results indicate that matrix interference can be excluded. Recovery of radioactivity in feces and urine was constant, and ranged from 85.0 to 91.8% (Table 1). At the lowest levels of radioactivity calculation of the statistical error demonstrated that the SD was less than < 1c.p.m. and the CV was 5.2 and 4.9% for feces and urine samples, respectively.

Clinical applicability

The radioactivity excretion profile in feces and urine after docetaxel infusion (210 mg) and an added tracer amount of [^{3}H]docetaxel is displayed in Figs 2 and 3, respectively.

During the first 72 h after i.v. administration (i.e. the period during which the patient was admitted in the hospital), the total percentage of recovered radioactivity was 60.41 (55.79% in feces and 4.62% in urine), which is within the expected range considering the time interval of sample collection. Loss of added [^{3}H]docetaxel due to adhesion to the infusion bag or administration set was negligible for both high (100 mg/m^2) and low (10 mg/m^2) doses of docetaxel (0.3% or less).

Discussion

We have here described the validation of a combustion method to quantify radioactivity levels in human feces and urine collected after i.v. administration of a tracer amount of [^{3}H]docetaxel added to a docetaxel infusion. The described method is rapid, specific, precise, has a recovery at least of 85% or above and allows sample aliquots of approximately 1.5 g (1:3 w/v homogenized feces or 2 ml urine) to be processed, which is more than previously described for analytical procedures using a non-combustion sample pre-treatment method (i.e. 200 µl feces homogenate 1:10; 20 mg dried feces; www.packardbioscience.com) [2] and, as such, lower levels of radioactivity can be detected. Indeed, when we analyzed our QC samples according to the previously described non-combustion method [2] the lowest QC level (for both matrices) could not be quantitated (data not shown); recovery for the other two QC levels was in the same range as we found for the combustion method. Furthermore, samples were clear, yet never completely colorless after sample preparation and chemiluminescence correction was required.

The described combustion method was applied to samples collected from a cancer patient who received a docetaxel infusion with a tracer amount of [^{3}H]docetaxel added. Recovered radioactivity was within the expected range taking into account the period of sample collection (i.e. 72 h). The method can therefore be applied to assess the influence of ketoconazole on the excretion profile and mass balance of docetaxel and its metabolites in feces and urine samples.

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