

**A COMPETITIVE ENZYME LINKED IMMUNOSORBENT
ASSAY FOR THE DETERMINATION OF DIMINAZENE
RESIDUES IN ANIMAL TISSUES****Karanja WM^{1*}, Mdachi RE¹, Ngeranwa JN² and JI Murungi³****Wycliff Karanja**

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ABSTRACT

The importance of ensuring food safety through the reduction of chemical residues in our food supply cannot be overemphasized. Food safety remains a major challenge confronting contemporary society. To ensure wholesomeness of food of animal origin, the level of drug residues must be below the maximum residue limits (MRLs) set by World Health Organization (WHO) and Food and Agriculture Organization (FAO). This calls for cost effective and efficient analytical methods for both quality assurance and monitoring. Diminazene aceturate is one of the few treatment drugs for animal trypanosomosis in the market. Because of its wide use in food producing animals, unwanted residues may be a risk to consumers. A competitive enzyme-linked immunosorbent assay (cELISA) for determination of diminazene residues in edible animal tissues after extraction in 0.1 M borax pH 9.7 is described. The assay has advantages of speed, high throughput and lower cost of analysis compared to the other conventional methods. The assay uses rabbit anti-diminazene polyclonal antibodies bound on a 96-well microtiter plate. Horseradish peroxidase-labeled diminazene and diminazene in a test sample were allowed to compete overnight at 4°C for the limited number of antibodies bound on the microtiter plate. After six washes with buffer, enzyme activity was determined by adding tetramethyl-benzidine and hydrogen peroxide as substrate. The assay detection limits for diminazene were 2.4 ng/g in muscle, 2.5 ng/g in liver and 2.2 ng/g in kidney while limits of quantification were 7.2 ng/g, 7.5 ng/g and 6.6 ng/g respectively. The recoveries for muscle liver and kidney spiked with 5 ng/g were 78%, 77% and 80% respectively while for 1,000 ng/g were 74%, 76.% and 84% respectively. The within-and between assay coefficients of variation (CV) were 2.4% and 15.5% respectively while assay specificity was above 99.9%. It is concluded that as a result of the good recoveries, high specificity and repeatability, the method could be used in the determination and monitoring of diminazene residues in tissues. These activities aimed at ensuring the safety of food of animal origin could play a major role in enhancing consumer confidence in these products which are very essential for health.

Key words: Diminazene, Residues, cELISA, Animal tissues, Trypanosomosis

INTRODUCTION

Antimicrobial drugs have been used for over fifty years in food producing animals to control, prevent and treat infections resulting in increased growth, feed efficiency and increased economic return to the farmers. Currently approximately 80% of all food producing animals receives medication for part or most of their lives [1]. The high reliance on antimicrobials in livestock farming may result in residues in edible animal products above the statutory levels.

Residues in foods of animal origin constitute a major food safety concern in the contemporary society.

Apart from the evidence associating them with specific health hazards, residues in foods also create trade disputes [2]. Analytical methods to monitor veterinary drug residues in animal tissues are essential to help protect human health, support the enforcement of laws and regulations and ensure safe and saleable food products for both local and international trade.

Diminazene aceturate is an effective drug used in the treatment of trypanosomiasis a disease caused by protozoan parasites known as trypanosomes. This drug is used in cattle, sheep, horses, dogs, rabbits and even chicken at recommended intramuscular dose of 3.5 mg/kg [3]. A higher dose of 7 mg/kg has been used where resistance to diminazene has been observed [4]. The use of higher than the recommended dose may pose a problem of residues in meat since the current withdrawal period was set based on a treatment dose of 3.5 mg/kg. Other causes of high residue levels include contravention of withdrawal period and route of administration of the drug. Although toxicity of diminazene to human is not well documented, diminazene has been shown to cause mutation to yeast cells of *Saccharomyces cerevisiae* and river buffalo cells [4, 5]. These findings point to an underlying health risk posed by diminazene residues in meat. To mitigate the risk factors, the FAO/WHO joint expert committee has come up with maximum residue levels (MRLs) for diminazene in edible animal tissues (500 µg/kg, 12,000 µg/kg and 6,000 µg/kg of muscle liver and kidney respectively) [6]. To monitor the compliance with the set MRLs, an efficient screening method for determination of diminazene residues in meat and meat products is desirable. A number of methods have been described for determination of diminazene in animal tissues and body fluids. These methods include Gas chromatography and mass spectrometry with a limit of detection (LOD) of 0.1 µg/ml and thin layer chromatography (TLC) with LOD of 0.01 µg/ml for plasma and 0.05 µg/g of tissue [7, 8].

High performance liquid chromatography (HPLC) with detection limits of 0.05 µg/g has been used in the pharmacokinetic studies of diminazene and monitoring of milk for diminazene residues at the 25 µg/kg level with a limit of quantification of 10 µg/kg [9, 10, 11,12]. Radiometric methods with detection limits of 0.028 µg/ml plasma and 0.18 µg/g tissue have been used to study the disposition of diminazene in cattle [13, 14, 15]. The method of liquid chromatography coupled with mass

spectrometry has been employed in the detection of diminazene residues in milk with sensitivities of 1 µg/kg [16].

Enzyme linked immunosorbent assay has also been employed in the determination of diminazene levels in cattle serum with a detection limit of 0.8 µg/l [17]. Most of these methods except those based on ELISA are lengthy, have low throughput, low sensitivities and require more sophisticated instruments [18]. No ELISA method has been reported previously for diminazene residues in tissues. The conditions for the diminazene tissue ELISA cannot be extrapolated directly from those determined for serum due to the differences in serum and tissue matrix. The main difference between ELISA using serum or tissue samples is the sample preparation for while no further clean up is required for serum, this is the critical step for tissues. This paper describes an enzyme-linked immunosorbent assay (cELISA) for diminazene residue in tissues. The cELISA is based on polyclonal antibodies and determines diminazene to a level of 2.5 ng/g tissue. Goat muscle tissues fortified with diminazene at levels ranging from 5 ng to 1000 ng per gram were evaluated with the cELISA.

MATERIALS AND METHODS

Chemicals and materials

Diminazene diacetate (98.7% pure) was obtained from Ceva Sante Animale Libourne Cedex France while N-hydroxy-2-5-pyrrolidinedione (N-Hydroxysuccinimide), Polyoxyethylenesorbitan monolaurate (Tween 20), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide, peroxidase enzyme type VI from horseradish, human transferrin, glutaraldehyde and sephadex G25 were all obtained from Sigma-Aldrich St Louis MO USA while Freud's complete and incomplete adjuvants were from DIFCO, Detroit, USA. Buffer salts of analytical grade were obtained from various sources which included British Drug House (BDH), Poole, England; Panreac, Barcelona, Spain and Associated chemical enterprises, Republic of South Africa.

Experimental animals

All protocols and procedures used in animal studies were reviewed and approved by the Trypanosomiasis Research Centre of Kenya Agricultural Research Institute (KARI-TRC) institutional animal care and use committee.

Two rabbits of the New Zealand white breed were acquired from the small animal breeding unit of KARI-TRC and housed in individual cages. They were fed on rabbit pellets (Belfast feeds Ltd, Nairobi, Kenya) supplemented with green vegetables and water *ad libitum*. The animals were allowed 2 weeks acclimatization period before use in the experiment.

Preparation of diminazene conjugated with transferrin for production of polyclonal antibodies and diminazene conjugated with horseradish peroxidase (HRP) for ELISA

The diminazene–transferrin conjugate was prepared as antigen using a modified two stage glutaraldehyde coupling method [19]. Briefly to a 1% w/v human transferrin in 0.1M phosphate buffered saline (PBS) pH 7.4 was mixed with 20 µl of 4% v/v glutaraldehyde in distilled water. The mixture was incubated overnight at 4°C with mixing. The reaction was stopped with 1m L-lysine and excess glutaraldehyde removed by passing mixture through a Sephadex G25 column. One milliliter of 0.25% w/v diminazene water solution was added to the activated transferrin and after coupling overnight at 4°C the conjugate was purified using a Sephadex G25 column.

To prepare the diminazene- horseradish peroxidase conjugate four solutions were first prepared and then mixed as described below:

Solution 1- 20 mg diminazene acetate in deionized distilled water to which was added 0.25 ml pyridine.

Solution 2- 1 mg N-hydroxysuccinimide ester (NHS; Sigma H-7377) in 0.1 ml dimethyl sulfoxide.

Solution 3- 10 mg of N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride carbodiimide (Sigma-Aldrich; E-7750-5G).

Solution 4- 20 mg horseradish peroxidase (HRPO; Sigma P-8375) in 0.5 ml deionized and distilled water

The four solutions were mixed together and stirred overnight at room temperature. To purify the conjugate, a six centimetre dialysis tubing Visking size 2.18/32” (Medicell International 239 Liverpool Road, London) was boiled in deionized distilled water for 15 minutes. The conjugate was dialysed in this tubing for seven hours in distilled water. The dialysed conjugate was then passed through a Sephadex G25 column and eluted with normal saline into a bijou bottle. The eluted conjugate was aliquoted into two Eppendorf tubes and to each added a 100 µl of dextran coated charcoal in distilled water. The tubes were centrifuged at 12,000 g for 30 minutes using a Hawksley hematocrit centrifuge (Hawksley and sons Ltd, Marsborough road Lancing, Sussex, England Cat.No.01300-00). The supernatant was decanted and an equal volume of glycerol added before storing the conjugate at -20°C.

Production of the polyclonal antibodies

The eluted conjugate for immunization was diluted three times in normal saline and 2 ml of this solution mixed with 4 ml of complete Freund’s adjuvant and three rabbits immunized with intradermal injections of 0.5 ml at four different sites. Subsequent booster injections of conjugate in incomplete Freund’s adjuvant were administered at four weeks intervals for 20 weeks. The rabbits were test bled every two weeks for the first 14 weeks and then weekly, up to time they were sacrificed 22 weeks after the first immunization

Hyperimmune serum and diminazene-enzyme conjugate titration

Hyperimmune serum collected 21 weeks after the primary immunization of the rabbits was used in a checkerboard titration with the diminazene-enzyme conjugate to determine their optimum dilutions. Microtitre plates (96 well Immulon 4 Dynatech labs, Chantilly, USA) were coated with hyperimmune serum, serially diluted in 0.1 M carbonate bicarbonate buffer pH 9.2 from 1/100 to 1/204,800. The antiserum was titrated with diminazene-enzyme conjugate serially diluted from 1/1000 to 1/128,000 in blank tissue PBST extract. The percentage competitions of pure diminazene at a concentration of 0.5 ng per well and the enzyme conjugated diminazene were determined in duplicate for antiserum dilutions of 1/4000 to 1/32,000 and conjugate dilution of 1/8,000 to 1/64,000.

Generation of standard curves

Two grams of liver, kidney and muscle tissue chopped into small pieces were weighed in 50ml plastic tubes and 12ml of borax buffer added. The tissues were homogenized using an Ultra-turax tissue homogenizer (Janke and Kunkel IKA-Labortechnik). The mixture was centrifuged at 1,800 g using a Megafuge 1.0 (Hereus sepatech) and supernatant decanted into clean 50 ml tubes. The supernatant was used in the preparation of diminazene standards ranging from 500 ng/ml to 0.025 ng/ml of tissue extract.

The competitive enzyme-linked immunosorbent assay (cELISA)

A 100 µl of hyperimmune serum diluted with coating buffer was pipetted into wells of microtitre plate (96 well Immulon 4[®] Dynatech labs, Chantilly, USA) and incubated at 4°C overnight and thereafter frozen to -20°C. Plates were then thawed and washed five times using 0.3 ml/well of phosphate buffered saline containing Tween 20 (PBST). A 100 µl of diminazene standard or tissue extract and the diminazene-enzyme conjugate were added to each well. The plates were incubated overnight at 4°C. The washing step was repeated to remove the unbound compounds. A 100 µl of substrate solution comprised of hydrogen peroxide and a chromophore, 3,3,5,-tetramethylbenzidine (TMB) was then added to each well. The enzymatic reaction was stopped after 10 minutes by addition of 100µl of 1M orthophosphoric acid. Absorbance (OD) values were measured at 450 nm using a 96-well microtitre plate reader (Immunoskan PLUS Type 314 of Labsystems, Finland). The concentrations of analyte in samples were read directly from the calibration curve generated in blank tissue buffer extract.

Determination of limits of detection and quantification

A representative sample of 50 g diminazene free liver, muscle or kidney tissue was weighed into a 50 ml tube and chopped in small pieces. Twenty sets of one gram tissue were sub sampled and extracted with 6 ml borax buffer. The OD of the extracts was measured and mean and standard deviation calculated. The concentration interpolated from the standard curve corresponding to the mean OD minus three standard deviations was taken as the limit of detection. The limit of quantification was the concentration corresponding to three times the limit of detection [20]

Determination of assay accuracy

To determine accuracy of the assay each set of 5 one-gram blank muscle liver and kidney tissue samples were extracted with 6 ml borax buffer pH 9.7 and 0.1M. A volume of 10 ml muscle blank extract was spiked with diminazene at a concentration of 5 ng/ml and 10 ng/ml. The spiked tissue was replicated on the ELISA plate 15 times for each concentration of the muscle extract and 10 times for non-spiked blank extracts of all three tissues and assayed quantitatively against diminazene standards.

Recovery of diminazene from tissues using 0.1 M borax pH 9.7

Diminazene was extracted with different volumes (1-20 ml) of borax from tissue samples spiked at concentrations ranging from 5 ng/g to 1,000 ng/g. Six sets of one gram of tissue were weighed, chopped into small pieces using a pair of scissors and then spiked with a predetermined amount of diminazene (5 ng/g to 1,000 ng/g). The samples were mixed and left standing for five minutes before adding a volume of borax solution ranging from 1.5 ml/g to 10 ml/g. The samples were mercerated using an ultra turax tissue mercerator, shaken for one minute and centrifuged for 10 minutes at 1,800 g. The supernatant was decanted, diminazene concentrations determined using standards prepared in the blank tissue extract and percentage recoveries calculated. The variation of recovery with extraction volume was determined using one way ANOVA and t-test.

Assay precision (intra and inter-assay variation)

Diminazene standards of 5 ng/ml and 10 ng/ml were prepared in PBST liver extract. Each standard was replicated fifteen times on microtitre plates and assay repeated for three days. The concentrations were determined and variations within the plate (intra-assay) and between assays (inter-assay) determined by one-way ANOVA using the method of Rodbard as illustrated for the 10 ng/ml [21]

SD within samples for r replicates = mean of SD/ $r = 1/15 = 0.258$

SD between assays = $[(\text{mean SD})^2 / r + \text{SD}_b^2]$ where $\text{SD}_b = [(\text{SD of assay means})^2 - (\text{mean SD})^2 / r]$

$\text{SD}_b = [(1.64^2) - (1^2/15)] = 1.619$

Therefore SD between assays = $(1^2/15 + 1.619^2) = 1.639$

CV(%) within assay = $0.258/10.56 \times 100 = 2.44$

CV(%) between assays = $1.64/10.56 \times 100 = 15.5$

RESULTS**Optimum titres for diminazene-enzyme conjugate and hyperimmune serum**

Two rabbits had very poor immuno responses giving a titre of 1/100 even after receiving 4 booster injections. However, the antisera of one of the rabbits gave ODs above 0.7 at a dilution of 1/16,000.

The highest antiserum titre was obtained in the 21st week after primary immunization and was used in the optimization procedures. The dilutions of antiserum lower than 1/8,000 with conjugate dilution lower than 1/64,000 gave absorbance values of one and above while antiserum dilutions lower than 1/16,000 resulted in percentage competitions of 50 and above as can be observed in Table 1. The optimum antibody and conjugate dilutions that gave highest competition (81.4%) and OD (0.951) close to one were 1/8000 as shown in Table 1.

Standard curves

Sigmoidal curves fitted with the four parameter logistic equation introduced by Healy and modified by Rodbard were obtained for the liver, muscle and kidney [19, 20, 21, 22]. The fitting was characterized by r^2 values above 0.998. The standard curves for liver muscle and kidney are shown in Figure 1

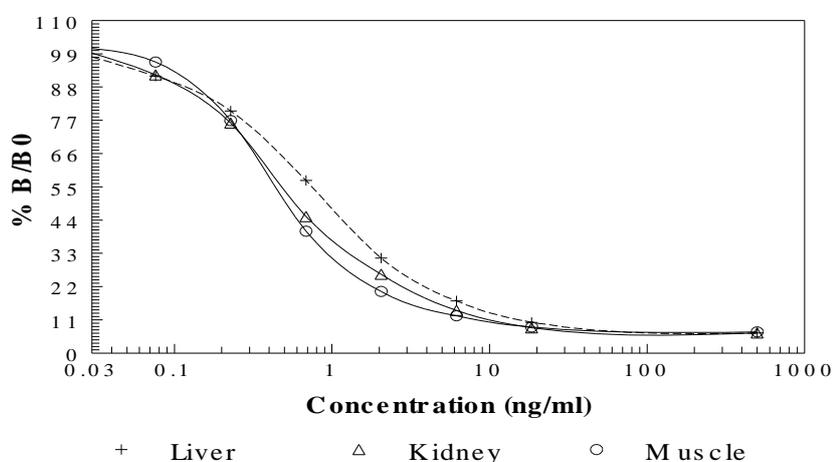


Figure 1: Diminazene standard curves for goat liver, muscle and kidney

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection for diminazene in goat tissues extracted in borax 0.1M, pH 9.7 obtained per milliliter of tissue extract were 0.808 ng/ml (2.42 ng/g), 0.831 ng/ml (2.493 ng/g) and 0.736 ng/ml (2.208 ng/g) for skeletal muscle, liver and kidney respectively. The limits of quantification (LOQ) D for goat muscle, liver, and kidney were 7.2 ng/g, 7.5 ng/g and 6.6 ng/g respectively.

Percentage recovery of diminazene from spiked animal tissue

The recovery of diminazene using 0.1 M borax pH 9.7 and worked out as mean and standard deviation (SD) was 77.6% ± 16.9%, 80.2% ± 6.71%, 77.3% ± 8.22% for 5 ng/g spiked muscle, kidney and liver respectively and 79.2% ± 8.3%, 84.1% ± 9%,

and $75.5\% \pm 6.7\%$ for 1,000 ng/g spiked muscle, kidney and liver respectively. The volume of buffer used in the extraction varied with the amount of diminazene spiked into the sample. This can be deduced from the results shown in table 2.

Assay accuracy

Assay accuracy is the closeness of agreement between the true value and the mean result obtained by analysis of fortified samples with known amounts of diminazene expressed as a percentage. In this study accuracy was determined for diminazene concentrations of 5 ng/g and 10 ng/g of tissue. For duplicate plates the mean percent closeness for fifteen replicates were $94.1\% \pm 7.4\%$ with CV of 7.2% for the 5 ng/g while for 10 ng/g the values were $98.5\% \pm 11.5\%$ with CV of 10.42%. The assay was also capable of detecting a blank sample as having zero drug concentration except in few instances where the blank had a reading. All the readings for blank samples were, however, below detection limit.

Intra-assay and inter-assay variation (precision)

The intra-assay (within-assays) coefficient of variation (CV%) was found to be 2.44% and 2.24% for 5 ng/ml and 10 ng/ml sample respectively, while the inter-assay (between-assays) CV% was 15.5% and 15.2% for 5 ng/ml and 10 ng/ml respectively.

DISCUSSION

The method described here has advantages of high throughput, lower cost of analysis and cheaper instrumentation compared to other published methodologies for determination of diminazene residues in animal tissues. The other methods also use relatively large volumes of organic solvents [10, 13, 21 23]. They may also involve other more complicated procedures such as solid phase extraction. These extra procedures are laborious increase the cost of analysis and lead to reduction in recoveries. The extraction of diminazene from tissues for cELISA on the other hand may require as low as 6 ml of a relatively cheap borax buffer and results in higher recoveries. The cELISA applied here was accurate, precise and specific for diminazene. These may be attributed to presence of high affinity of antibodies to diminazene and diminazene-enzyme conjugate of very high titre. A high dilution of immuno reagents also dilutes any unwanted matrix hence reducing interferences that could mask the analyte. Use of reagents at very high dilutions reduces their rate of consumption and minimizes the frequency of their preparation. The low immuno-responsiveness in the one of the rabbits is a phenomenon frequently observed in the production of polyclonal antibodies [24].

In order to optimize the extraction procedures different buffers were tested. The borax buffer at pH 9.7 and strength of 0.1 M proved to be the most effective when used in the correct volumes as shown in Table 2. The volume was shown to vary to a certain extent with the level of diminazene in the samples although an extraction volume of six milliliters per gram of tissue would be an ideal starting point. This phenomenon can be explained by the fact that at low concentrations large volumes would dilute extracted diminazene to levels below limit of detection. For more

concentrated samples larger buffer volumes would on the other hand disperse the matrices and reduce the force of attraction of diminazene to their surfaces.

The low limits of detection and quantification are expected for assays with high specificity. For all the substances tested the cELISA showed only slight cross reactivity (0.003%) with isometamidium chloride. The high specificity of over 99.9% obtained for the cELISA contributed to the low LOD and LOQ. The assay accuracy was above 94%. The percent error between the assay-determined value and that assigned was within the acceptable level of less than 20% for Enzyme immunoassays [25]. High assay specificity and accuracy for diminazene renders the assay usable for both qualitative and quantitative determination of diminazene. The low LOD and LOQ are an indication that the assay is capable of detecting diminazene at the MRLs. Assay coefficients of variation indicate that the precision of the assay (below 2.45 for intra-assay and less than 15.5% for between-assay) is good. The values are within the accepted range of the International Union of Pure and Applied Chemistry (IUPAC) for immunoassays [26]. For the range of concentrations used in the assay, the allowed maximum variations are 12.5% for intra-assay and 25% for inter-assay. The high precision for the method is an indication of assay robustness and minimal matrix interference. Use of very low volumes of organic solvents relative to other conventional methods minimizes the constraints of waste disposal especially in developing countries. The properties discussed render the assay suitable for routine monitoring and regulation of diminazene residues in meat.

However, it should be noted that further development of the method aimed at reducing the analysis time to a few hours is necessary. This would enhance the effectiveness of the assay especially for purposes of screening.

CONCLUSION

The ELISA described for diminazene in tissues of animals exhibited good specificity, precision, accuracy and low limit of quantification. The assay therefore, can be effectively employed in monitoring consumer exposure to diminazene residues and screening of edible animal tissues to ensure safety of these products before they reach the consumer. Most of the immunochemical reagents used in the assay are produced in-house and this minimizes reliance on imports. The disadvantages of importation are that apart from exorbitant cost, the reagents may also deteriorate during transportation. This method is especially suited to the developing countries since most use diminazene to treat livestock for trypanosomiasis.

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Table 1: Optical densities (ODs) of spiked sera (5 ng/ml) and corresponding percentage competition for bound antibody between diminazene-enzyme conjugate and 0.5 ng free diminazene per well of microtitre plate

Antisera	1/4,000		1/8,000		1/16,000		1/32,000	
	OD	Competition % ±SE	OD	Competition % ±SE	OD	Competition % ±SE	OD	Competition % ±SE
1/8,000	2.468	56.5 ± 1.8	1.827	58.3 ± 2.1	0.724	55.5 ± 2.4	0.348	57.8 ± 1.3
1/16,000	2.327	71.2 ± 2.1	1.686	72.3 ± 2.3	0.645	69.2 ± 2.1	0.278	60.9 ± 2.0
1/32,000	1.927	79.9 ± 2.5	1.438	79.6 ± 2.0	0.529	73.6 ± 1.6	0.234	60.6 ± 2.2
1/64,000	1.286	81.9 ± 2.1	0.951	81.4 ± 1.8	0.372	74.2 ± 2.1	0.167	50 ± 2.8

Mean ± SD

Table 2: Mean (n=6) recovery of diminazene from 1gram bovine skeletal muscle spiked at different concentrations of the drug and extracted with different volumes of 0.1 M borax pH 9.7 with standard deviation, the CV and Lsd are not necessary as they have the same role. So you chose to use either \pm sd or % CV and Lsd. The recovery and percent recovery may be the same! You can therefore use the percentage recovery only.

Standard ng/g	Conc./ml extract	Extraction volume	Recovery (ng)	Percentage recovery	Standard deviation
5	1.33	2.0	2.66	53.16	5.26
5	1.29	3.0 ml	3.88	77.60	16.93
10	2.17	2.0 ml	4.34	43.4	1.19
10	2.05	4.0 ml	8.19	81.93	7.98
25	3.99	4.0 ml	15.96	63.86	2.32
25	3.16	6.0 ml	18.93	75.73	4.12
50	7.82	4.0 ml	31.28	62.57	4.82
50	6.38	6.0 ml	38.30	76.59	6.60

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