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Spectrophotometric Assay for the Quantification of Plasma Ethanol Levels in Mice through Chromium-Ethanol Oxidation-Reduction Reaction

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Abstract

The quantification of blood/plasma ethanol concentration (BEC/PEC) is of great importance in experiments involving basic research, clinical studies, and bioethanol production. Traditional methods commonly used to measure BEC can be expensive and require high-cost equipment and qualified labor. The aim of this study was to develop a low-cost method that can be performed with simple infrastructure commonly available in research laboratories. For this, we developed a protocol to quantify PEC in mice, using the method of reduction of potassium dichromate by ethanol. However, this oxidation-reduction (redox) reaction is not specific to ethanol. Thus, the PEC was measured following a sequence of chemical reactions to eliminate the reductive interfering substances presented in the samples. Firstly, we evaluated the sensitivity of the dichromate reactive to ethanol and to different reducing substances found in the plasma, in order to determine which the main interfering substances are. Next, once the main interfering substances were determined in the dichromate reduction, plasma was assayed for PEC. First, mice received intraperitoneally (i.p.) saline (basal reading, 0% ethanol) or ethanol injections (0.5, 1, 2, 3, and 4 g/kg) and had their plasma collected. After plasma deproteinization and plasma glucose oxidation, it was mixed with the dichromate/acetic acid reactive, and then the products of the redox reaction were determined by the spectrophotometric method. Then, we determined the PEC with the same plasma samples using a commercial ethanol assay kit as a positive control. We found an excellent correlation between the administered ethanol doses and PECs in both the methods analyzed. The values of PEC found in the dichromate reaction method were similar to those obtained in the literature with the same ethanol doses, and to the commercial enzyme ac-

tivity assay. Therefore, despite the need for a background reading, this method can be successfully applied to determine PEC using low-cost chemical reagents.

Keywords

Non-Enzymatic Ethanol Quantification, Spectrophotometric Assay, Potassium Dichromate, Oxi-Reduction Reaction, Blood Ethanol Concentration

1. Introduction

The quantification of plasma or blood ethanol concentration (PEC/BEC) is of great importance in basic [1] [2] [3] [4] [5], clinical research [6], forensic reports [7] as well as in bioethanol production [8] [9]. In basic research, for instance, BEC provides more accurate information about differential responses in specific behaviors. It is used, for example, to compare sensitivity to loss of righting reflex among strains [10] and whether behavioral differences between ages in response to ethanol are due to differential ethanol pharmacokinetics [3].

Commonly used methods to quantify plasmatic ethanol including gas chromatography, enzymatic-oxygen rate alcohol analyzers, and commercially available colorimetric assay kits via enzymatic oxidation are expensive and/or require high-cost equipment and skilled labor. Therefore, it is of great relevance to develop lower cost alternatives that can also be performed with infrastructure commonly available in research laboratories and of easy acquisition.

Ethanol oxidation by potassium dichromate solution has been utilized to determine ethanol concentration in breathalyzers [11], in alcoholic beverages [12] [13], and in blood plasma [14]. In the later cases, however, the method is complex, involving distillation and titration. In an acidic solution, ethanol reacts with dichromate, and the hexavalent chromium Cr^{6+} is reduced to Cr^{3+} while ethanol is oxidized to acetic acid, changing the orange solution color to green. However, the chromium reduction from Cr^{6+} to Cr^{3+} can be induced by a variety of elements present in blood samples, making the reaction poorly specific for ethanol. Some authors tried to circumvent this limitation by using solvent extraction [9]. However, it provides relative values, given that ethanol is found in both polar and non-polar phases after solvent extraction. Despite the lack of specificity of Cr^{6+} reduction, we searched for alternatives to eliminate interferences (*i.e.* glucose, proteins, etc.) in the redox reaction between chrome and ethanol. One alternative is to transform and/or remove the main interfering substances that reduce chromium from the samples allowing the specific detection of ethanol. The interfering elements that remained even after these procedures could be regarded as background controls.

Therefore, in an attempt to obtain a method using potassium dichromate that is capable of accurately estimating ethanol concentration in the samples, the aim of this study was: 1) Compare the sensitivity of dichromate reactive by oxidizing the main nutrients and metabolites found in the plasma (such as glucose, glyce-

rol, lipids, vitamins, ketones, aldehydes, lactic acid, amino acids, and creatinine); 2) Perform chemical and physical steps in order to eliminate the main interfering substances that are capable of reducing Cr^{6+} to Cr^{3+}; 3) Verify a putative positive correlation between ethanol doses injected in mice and PEC, after submitting the samples to a sequence of chemical reactions, and 4) Compare the absolute values obtained from dichromate reaction with a control method, more specifically, the colorimetric method that uses the alcohol oxygenase enzyme.

2. Material and Methods

2.1. Animals

A total of 14 Swiss male mice, 80 days old, were maintained in polycarbonate cages (42 cm length × 28 cm width × 21.5 cm height), grouped in 4 - 5 mice per cage, with food and water *ad libitum* throughout the entire experiment, under controlled temperature (21˚C ± 1˚C) and the light/dark cycle of 12:12 h (lights on at 7:00 AM). The mice were allowed to acclimate to the experimental room one week before the initiation of the experiment. The pharmacological experiments were conducted in the daytime (between 8:00 AM and 12:00 AM). All the experimental procedures were conducted according to the guidelines of the Brazilian National Council for Control of Animal Experimentation (CONCEA), in accordance with the Brazilian National Law number 11,794 of October 8, 2008, Decree 6899 of July 15, 2009, after the approval from the Ethics Committee of Animal Use (Protocol 5520141020) of the Institute of Biomedical Sciences of the Universidade de Sao Paulo.

2.2. Ethanol Solutions

Ethanol (95% v/v, Merck, Rio de Janeiro, Brazil) was dissolved in saline 0.9% w/v sodium chloride to produce a solution of ethanol 20% v/v and was administered intraperitoneally (i.p.) at the doses of 0.5, 1, 2, 3 and 4 g/kg. The saline i.p. injections were administered with equivalent volumes of saline at 0.9% w/v.

2.3. Reagents and Chemicals

D-glucose, L-lactic acid, ascorbic acid, α-tocopherol, L-glutamine, L-glycine and creatinine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium dichromate, glacial acetic acid, formaldehyde, methanol, chloroform, ethanol absolute, and trichloroacetic acid were purchased from Labsynth® (Diadema, SP, Brasil). Glycerol was purchased from Promega (Madison, WI, USA). Heparin was purchased from Cristália Chemicals & Pharmaceuticals Ltda (Itapira, SP, Brasil). The Kit Saccharide Removal Kit (DSRK-500) was purchased from Bio-Assay Systems (Hayward, CA. USA). The Ethanol Kit Assay was purchased from GenWay Biothec, Inc. (San Diego, CA, USA).

2.4. Standard Curves of Analytes Using Dichromate Reactive

To determine the sensitivity of dichromate reactive to the different nutrients,

metabolites and ethanol, standard curves were performed, using 200 uL/well, in 96-wells ELISA microplates, and read in a microplate reader spectrophotometer. The blank readings comprise all the reagents except the analyte, substituted by the same volume of ultrapure water. All samples were assayed in duplicates. High analyte concentrations that deviate from the Lambert-Beer law were removed from the standard curves.

A volume of 80 μL from the stock solutions containing different concentrations of the analytes was transferred to glass test tubes containing 1 mL of potassium dichromate ($K_2Cr_2O_7$) 5% (w/v) and glacial acetic acid (proportion 1:3 v/v). The reaction took place in a water bath at 95˚C for 10 minutes, then the test tubes were cooled in tap water, and 200 μL from each tube were added into the microplates. The spectrophotometric reading was performed at 570 nm. The criterium adopted to choose the analytes was their respective abundance in plasma. Therefore, we chose the following substances for the standard curves: ethanol, glucose, lipids, glycerol, lactic acid, acetone, amino acids glutamine and glycine, creatinine, formaldehyde (representing the ethanol metabolite acetaldehyde), ascorbic acid and *α*-tocopherol.

To build standard curves of lipids, first, we performed the lipid extraction by the Folch method [15]. The Folch method allows the extraction of total lipids (esterified and non-esterified cholesterol, triglycerides, saturated and unsaturated fatty acids, phospholipids). Polytron homogenization of hepatic tissue (50 mg of tissue) was conducted in falcons containing chloroform/methanol (500 μL methanol/1000μL Chloroform), and after the addition of 400 μL of distilled H_2O, the samples were vortexed to extract the non-lipidic hydrosoluble components (proportion of chloroform/methanol/H_2O of 8:4:3 v/v). Following centrifugation (5 minutes, 1200 RPM, 4˚C), the solution was separated into two phases: the upper aqueous phase (chloroform/methanol/H_2O 3:48:47) and the organic lower phase (chloroform/methanol/H_2O 86:14:1) [15]. Using a Pasteur pipette, the organic phase in the bottom was transferred to new test tubes. Chloroform was added into the aqueous phase, vortexed, the centrifugation step was repeated, and the organic phase was transferred to the tubes containing the organic phase previously obtained. After evaporation of the organic phase (containing mainly chloroform), in room temperature, the resulting lipid pellets were weighted and dissolved in glacial acetic acid.

2.5. Determination of Plasma Ethanol Concentration Using Dichromate Reactive

2.5.1. Plasma Collection from Mice

Mice were randomly assigned to one of the two treatments: ethanol or saline The mice received intraperitoneal injections of saline (0.9%) or ethanol (diluted 20% v/v in saline 0.9% w/v) at the doses of 0.5 g/kg, 1 g/kg, 2 g/kg, 3 g/kg e 4 g/kg, and right after, returned to their cages. After 10 minutes the i.p. injection was applied, 500 μL of blood were collected from the tail in eppendorfs containing 25 I.U. of the anticoagulant heparin sodium and then, centrifuged at 2000 × g, for 10 minutes at 4˚C, to separate the plasma from the serum. The plasma (around

120 μL/animal) was stored at −20˚C, and a few days later, the assays were performed. The tail blood collection was the method chosen to collect blood from mice because it allows the easy obtention of high blood volumes (up to 500 μL).

2.5.2. Glucose and Protein Removal

The plasma samples were placed on crushed ice, and 100 μL of plasma were mixed with 10 μL of Reagent A, 50 μL of the Reagent B and 11.66 μL of Reagent C, from the Saccharide Removal Kit (DSRK-500). After incubation for 15 minutes, at room temperature, the mixture was centrifuged for 5 minutes at 14,000 RPM and 80 μL from a purple supernatant were transferred to eppendorfs containing 80 μL of TCA 50%, turning from a purple color to transparent. The mixture was centrifuged at 14,000 RPM, for 5 minutes, at 4˚C, to precipitate the proteins. Then, 100 μL from the clear supernatant were transferred to glass test tubes containing 1 mL of potassium dichromate ($K_2Cr_2O_7$) 5% (w/v) and glacial acetic acid (proportion 1:3 v/v). The reaction happened in a water bath at 95˚C for 10 minutes, then the test tubes were cooled in tap water. After that, 1 mL from each tube was transferred to new eppendorfs, and then centrifuged at 14,000 RPM for 20 minutes, resulting in an insoluble pellet. The volume of 200 μL from each tube was pipetted onto microplates. The spectrophotometric reading was performed at 570 nm.

2.6. Determination of the Background Reading Composition after Glucose and Protein Removal

As previously mentioned, we intended to detect the background interferences and how much the background absorption contributes to the total absorbance reading. For this, the percentage values of each interference present in the plasma were multiplied by the respective calibration curve slopes. Given the sensitivity of dichromate reactive to the substances and/or their abundance in the plasma, we chose glycerol, formaldehyde, lactic acid and total lipids as the main candidates to be investigated and conducted the protocol in the plasma collected from saline control animals, following the same procedure described above.

2.7. Positive Control for Ethanol Plasmatic Concentrations

The PEC values obtained with dichromate reaction were compared with values detected in the Ethanol Kit Assay (Genway Biotech, Inc.) using the same plasma samples. This method is based on ethanol oxidation by the alcohol oxidase enzyme. This enzymatic reaction generates acetaldehyde and H_2O_2, and the latter reacts with a probe to generate a pink color (λ_{max} = 570 nm). The assay was performed following the manufacturer's instructions. The standard curve was performed from 2 nmoles to 18 nmoles of ethanol per well, making proper dilutions of the plasma samples to lie within the linear range of the curve.

2.8. Statistical Analysis

The Pearson correlation coefficient R was calculated to determine the correla-

tion strength between the ethanol doses injected in the mice and the apparent plasmatic ethanol concentration. The R values and their respective levels of significance p were calculated by the Statistica 7.0 software (StatSoft, Inc., 2004). Determination coefficient R^2 was calculated to demonstrate the goodness-of-fit of the linear regression model to the standard curves data. The linear regressions, the equations of the curves and R^2 values were all performed and calculated by the Excel software (version 365) and GraphPad Prism 7.0 software. To verify if there is parallelism between the curves yielded by the dichromate reactive and by the ethanol assay kit (positive control), the F-test for comparing regressions was performed, calculated by the GraphPad Prism 7.0 software. The level of significance for all the statistical tests was set at 5% ($p \leq 0.05$).

3. Results

3.1. Standard Curve of the Analytes Generated by Dichromate Reactive

The slopes of the standard curves yielded from each analyte (**Figures 1-3**) indicate the main reductive substances that interfere in the chromium reduction.

3.2. Determination of PEC by Dichromate Reactive and by Ethanol Assay Kit

Background absorbance after glucose and protein removal

The absorbance values from the saline controls were: 0.008, 0.008, 0.006, 0.007,

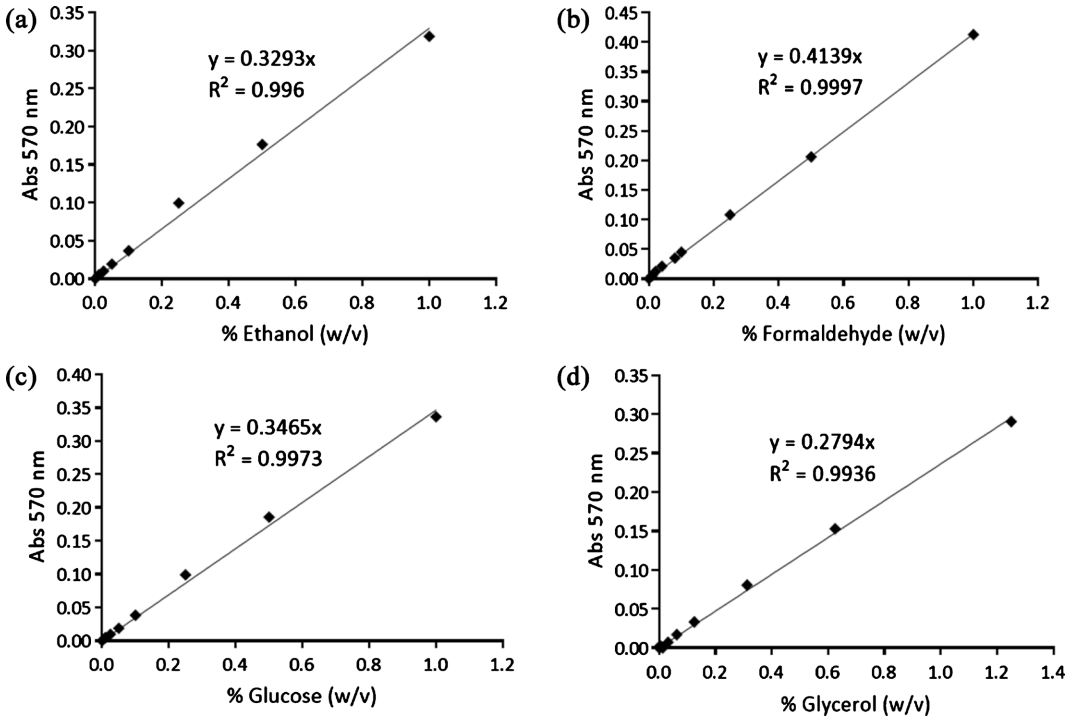

Figure 1. Standard curves of analytes, performed with dichromate reactive. (a) Ethanol (concentrations from 0.01% to 1% w/v); (b) Formaldehyde (concentrations from 0.01% to 1% w/v); (c) Glucose (concentrations from 0.01% to 1% w/v); (d) Glycerol (concentrations from 0.01% to 1% w/v).

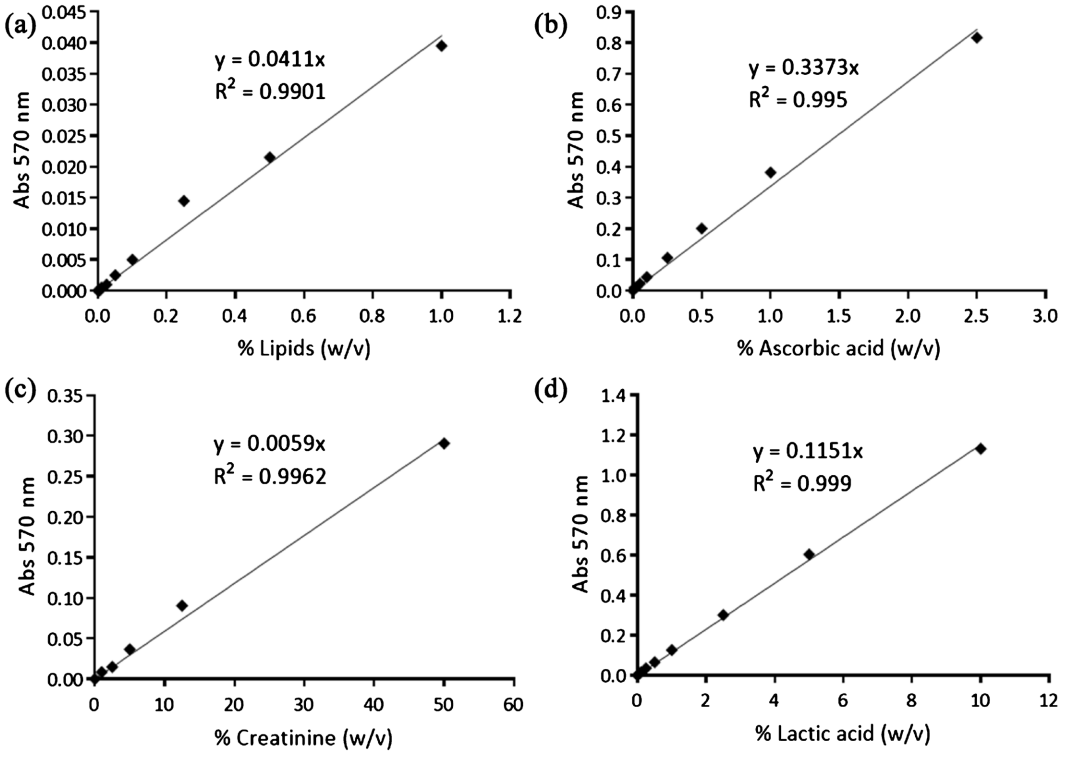

Figure 2. Standard curves of analytes, performed with dichromate reactive. (a) Lipids (concentrations from 0.01% to 1% w/v); (b) Ascorbic acid (concentrations from 0.01% to 2.5% w/v); (c) Creatinine (concentrations from 1% to 50% w/v); (d) Lactic acid (concentrations from 0.01% to 10% w/v).

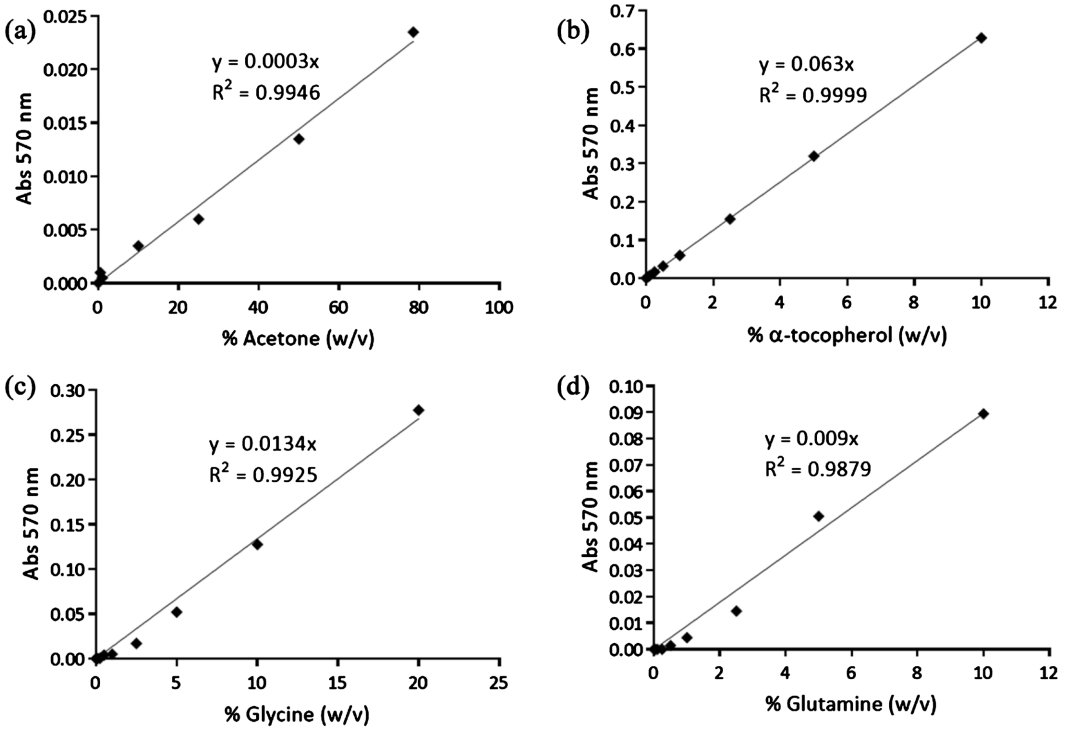

Figure 3. Standard curves of analytes, performed with dichromate reactive. (a) Acetone (concentrations from 0.5% to 78.5% w/v); (b) *α*-Tocopherol (concentrations from 0.025% to 10% w/v); (c) Glycine (concentrations from 0.025% to 20% w/v); (d) Glutamine (concentrations from 0.025% to 10% w/v).

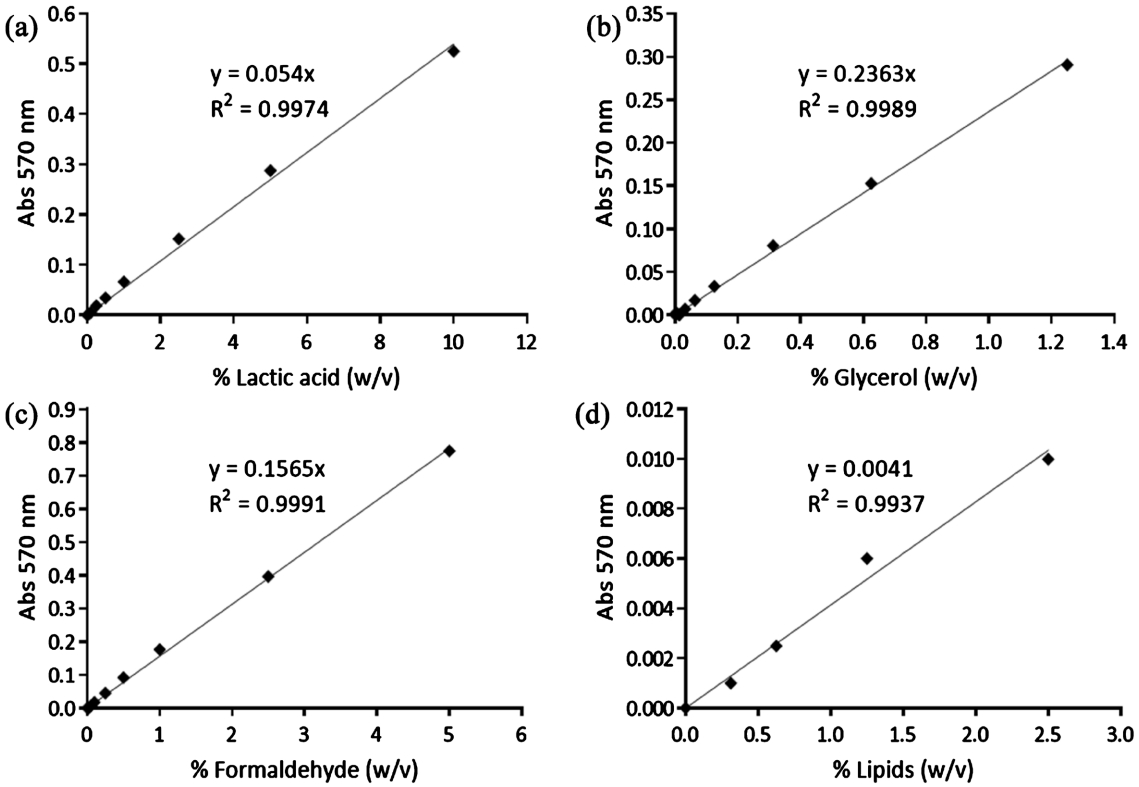

Figure 4. Standard curves of analytes, performed with dichromate reactive, after applying the same reagents and physical processes to eliminate glucose and protein. (a) Lactic acid (concentrations from 0.01% to 10% w/v); (b) Glycerol (concentrations from 0.001% to 1.25% w/v); (c) Formaldehyde (concentrations from 0.025% to 5% w/v); (d) Lipids (concentrations from 0.3% to 2.5% w/v).

0.0055, 0.006, 0.006, 0.006, 0.0065, resulting in a medium value of 0.0065. The absorbance values from each dose were subtracted from the value of 0.0065.

We can deduce that the main background absorbances come from the total lipid (whose plasmatic concentration is around 0.26%, giving an absorbance of 0.001), lactic acid (whose plasmatic concentration is around 0.036%, giving an absorbance of 0.002) and glycerol (whose plasmatic concentration is around 0.00138%, giving an absorbance of 0.00032). Therefore, summing these values, we have an absorbance of 0.0034, which is about half of the value compared to those obtained from our background controls (medium of 0.0065).

As depicted in Figure 5, the dichromate method provided a positive correlation between the ethanol doses administered and the plasma ethanol concentration ($R = 0.9956$; $p = 0.0003$). A positive correlation between the ethanol doses administered and the PEC was found with Ethanol Kit Assay as well ($R = 0.9896$; $p = 0.001$). Figure 5 also demonstrates the similarity of PEC between the non-enzymatic reaction using the dichromate method and the enzymatic reaction (positive control) using alcohol oxidase from the Ethanol Assay Kit. The F-test for regression comparison demonstrates that both lines in Figure 5 present no significant difference between their slopes ($F_{1,6} = 0.0902$; $p = 0.7741$), and no difference between their Y intercepts ($F_{1,7} = 1.449$; $p = 0.2679$).

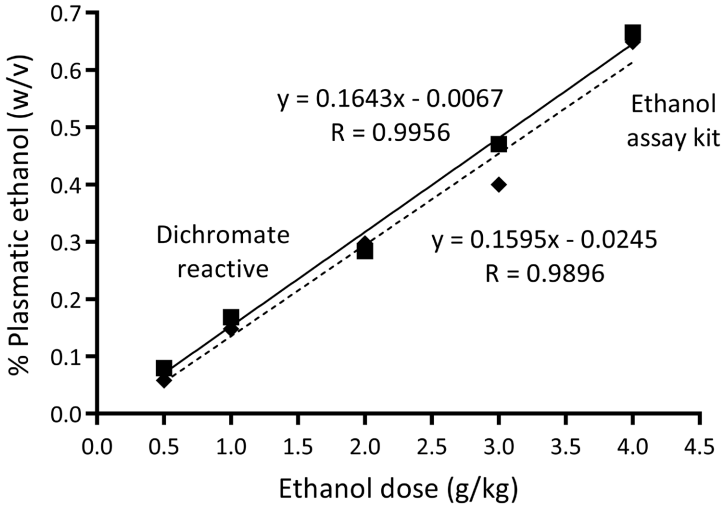

Figure 5. Correlation between the i.p. injection of ethanol (doses from 0.5 to 4 g/kg) and their respective ethanol plasmatic concentration (g/100 mL). Solid line: Linear regression with data (squares) obtained from the dichromate reactive and subtracting from a background reading taken from an average of saline injected mice. Dashed line: Linear regression with data (diamonds) obtained from the same samples but using ethanol assay kit (positive control).

4. Discussion

In the present study, the main findings can be summarized as follows: 1) Dichromate reactive, after controlling background readings, produces a strong linear correlation between ethanol dose administered and PEC; 2) The PECs obtained by dichromate reactive are similar to those previously shown in the literature; 3) The dichromate reactive and the standard colorimetric method via alcohol oxidase enzyme yield similar PECs for a wide range of ethanol doses.

Taking into account the slope values of the curves of each substance analyzed, we found that the dichromate reactive method presents higher sensitivity for ethanol, formaldehyde, ascorbic acid, glucose, and glycerol (slopes values of 0.3293, 0.4139, 0.3373, 0.3465 and 0.2794, respectively); median sensitivity to lactic acid (slope value of 0.1151); low sensitivity to *α*-tocopherol, lipids and glycine (slope values of 0.0133, 0.063, 0.0411 and 0.0134, respectively); and very low sensitivity to acetone, glutamine, and creatine (slope values of 0.0003, 0.009 and 0.0059, respectively). Substances such as ketone bodies (plasmatic concentration of acetoacetate in fasting: 0.001%) [16], *α*-tocopherol (plasmatic concentration of 0.0017%), creatinine (plasmatic concentration of 0.0001%) [17] and amino acids glutamine (plasmatic concentration of 0.008%) and glycine (0.002%) [18] do not represent important interferences in the assay due to the low sensitivity of dichromate reactive to detect these substances and low plasma concentrations. According to our standard curves and their respective plasma concentrations described in the literature, we determined which substances are likely responsible for the noise reading. This noise reading comes, in part, from lactic acid, lipids, glycerol, but also other substances we could not determine.

The ethanol metabolite acetaldehyde, catalyzed by the enzyme alcohol dehydrogenase, is found in low concentrations in the blood. In a previous study, the blood acetaldehyde concentration found was 0.00019%, 20 minutes after mice received an i.p. injection of 1.5 g/kg of ethanol [19]. This acetaldehyde concentration increased progressively until 2 hours after the injection—reaching almost 4 times the value measured 20 minutes after the ethanol administration—and then, fell progressively [19]. We indirectly demonstrated with formaldehyde reaction, that plasma acetaldehyde does not interfere in the absorbance. The lipids tended to have low oxidation potential by dichromate reactive (slope value of 0.0411). However, lipids concentrations in plasma are high enough (0.26%) [20] to interfere in the reading, as it is depicted in **Figure 4(d)**. Analyzing the standard curve (**Figure 4(a)**), lactic acid seems responsible for almost one-third of the background reading. Therefore, considering the abundance in the blood plasma of each substance and their respective capacity to reduce Cr^{6+} to Cr^{3+}, we conclude that, after protein and glucose removal, the main interferences in the chromium redox reaction are lipids and lactic acid.

Previous studies demonstrated that the plasmatic variation among individuals of glycerol, free fatty acids and triacylglycerol levels are very small in albino Swiss mice, as well as along 24 hours of fasting [21]. The basal lactate levels have also been shown to be very stable in the resting state, except after exercise [22]. However, when lactate is injected intraperitoneally in mice, it returns to its basal level as soon as 30 minutes after its injection [23]. Therefore, the background values are trustworthy for two reasons: firstly, because it gives a very low background value, and secondly, because it involves substances with low susceptibility to intraindividual variations.

As shown in **Figure 5**, we have observed a strong positive correlation between i.p. ethanol dose and the apparent PEC ($r = 0.9956$). The absolute values are equivalent to those previously obtained from the literature [2] [20] [24]. It is worth mentioning that PECs found in previous studies, [2] [20] [24] [25] used similar intervals (4 - 15 minutes) between the ethanol injection and blood collection for ethanol quantification. The positive control we have performed using the Ethanol Kit Assay—whose method is based on ethanol oxidation by the enzyme alcohol oxidase—yielded remarkably similar plasmatic ethanol values (**Figure 5**) when compared to the dichromate method. **Figure 5** also depicts a high level of parallelism between the curves. Despite the limitation of demanding background values, our method has an advantage over the Ethanol Kit Assay: our method comprises a wide range of ethanol dosages using the same plasma volume. It detected PEC from the very low dose of 0.5 g/kg i.p., which produces no visible behavior effect on the animal, to doses that induce locomotor stimulation (2 g/kg) or sedation/loss of righting reflex (4 g/kg). Therefore, in opposition to the Ethanol Kit Assay, there is no need to foresee the dilution factor necessary to obtain the sample absorbance value within the standard curve linearity. In addition, the linearity of the standard curves obtained from the commercial as-

say kits comprises narrow limits of ethanol concentrations.

The present method can conveniently be used to measure PEC. The main limitation is the need for correction for some of the interferences. The advantages over gas chromatography and ethanol assay kit are: 1) the relation between ethanol doses and PEC is linear in a wide range of doses, without the need for dilutions, such as in the colorimetric kit; 2) it can be performed in laboratories with minimal infrastructure using low-cost reagents. Further modifications in the assay procedure for a better readout might be developed in the future to quantify ethanol in different sources of biological samples.

Acknowledgements

Marcos Brandão Contó performed all the pharmacological and chemical assays, participated in the conceiving and the design of the research, performed the statistical analysis, and wrote the manuscript. Rosana Camarini participated in the conceiving of the research and writing. The authors read and approved the final manuscript. Marcos Brandão Contó was the recipient of a fellowship from CAPES. Funding for this study was provided by grant #2018/05038-0, from São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP). The authors would like to thank Priscila Marianno for her technical assistance with the blood collection from mice. Rosana Camarini is Research Fellow of CNPq (Conselho Nacional de Desenvolvimento Científico, "National Council for Scientific and Technological Development").

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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