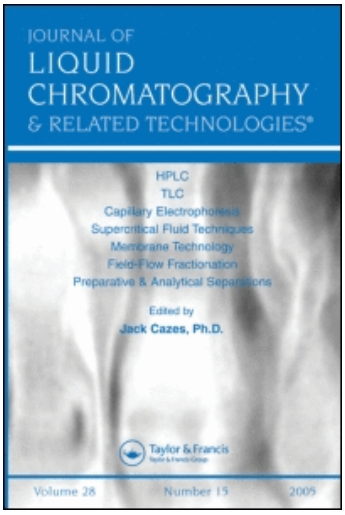

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STATISTICAL EVALUATION OF AGREEMENT BETWEEN HPLC AND COLORIMETRIC METHODS FOR ANALYSIS OF ALLANTOIN IN RUMINANTS' URINE

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ABSTRACT

In this study we compare an HPLC method for the determination of allantoin developed by us [1] with the traditional colorimetric method according to the Rimini-Schryver reaction modified by Fujihara [2]. Results were compared by using product-moment correlation ($r = 0.962$) as a measure, and by using the interclass correlation as the correct statistic for assesing agreement between both of them ($r_1 = 0.957$, lower limit = 0.916) for a 95% of confidence interval.

INTRODUCTION

Allantoin is one of the end and appears to be quantitatively the most important product of purine catabolism in ruminants [3]. More recent studies give further support to the usefulness of allantoin excretion in the urine as a possible indicator of microbial protein flowing to the small intestine [4].

Several analytical methods have been described for the determination of allantoin in biological fluids. The traditional colorimetric analysis of allantoin in urine is based on the Rimini-Schryver reaction described by Young and Conway [5]. Lindberg and Chen described a method that adapted this reaction to the Technicon Autoanalyzer [6-7]. More recently HPLC methods have been developed [1][8-9].

In this work, we described a comparative study of the allantoin determination in sheep urine by RP-HPLC method [1] and by colorimetric method according to the Rimini-Schryver reaction modified by Fujihara [2].

EXPERIMENTAL

Chemicals

Allantoin was obtained from Sigma Chemical Co. (St. Louis, Mo, USA) and used without further purification. Phenylhidrazine, potassium hexacyanoferrate (II), monobasic potassium phosphate, sodium hydroxide and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Methanol of HPLC grade was obtained from Carlo Erba Milan, Italy). Water was previously distilled and purified with a Milli-RO15 reagent grade system (Millipore, Bedford, MA, USA).

Urine samples

Urine was collected daily under toluene in metabolic crates. After removing the toluene, urine was stored at -20°C until analyzed. Urine samples were centrifuged and filtered through Millex-HV 0.45 μm pore size filter (Millipore) and diluted 10-fold (HPLC method) and 50-fold (colorimetric method) with deionized water before analysis. 20 μl of filtrate were injected into the HPLC column and 5 ml were used in the colorimetric technique. Urine samples were stable for several weeks when stored at -20°C.

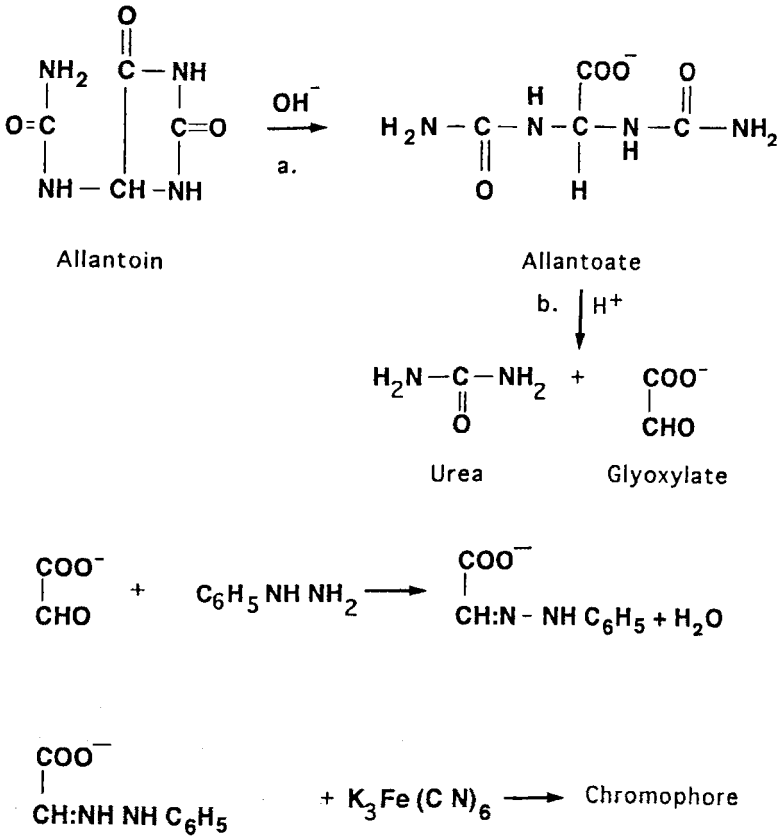

Fig 1.- Degradation of allantoin to urea and glyoxylate by alkaline hydrolysis (a.) and by acid hydrolysis (b.), and the subsequent formation of the chromophore.

ANALYTICAL PROCEDURES

Colorimetric Method

Analyses were performed on a Spectronic 2000 (Bausch & Lomb, Rochester, NY, USA) and were based on the Rimini-Schryver reaction (Fig.1) modified by Fujihara [2].

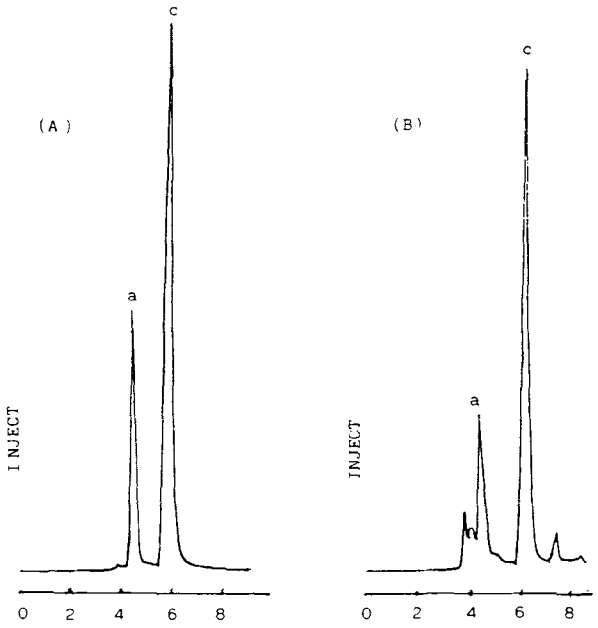

Fig 2.- HPLC separation. (A)-allantoin (60 μg/ml) and creatinine (40 μg/ml) standard solutions. (B)-urine sample. HPLC conditions: Nova-Pak C18 [300 x 3.9 mm (i.d), 4 μm particles] column; mobile phase: potassium phosphate buffer (10 mM, pH 4.0); flow rate 0.5 ml/min; column temperature 25 °C; detector wavelength 218 nm. Allantoin (a), Creatinine (c).

Chromatografic Method

Analyses were carried out on a Waters 600E (Waters Assoc., Mildford, MA, USA) equipped with a Waters 484 UV detector. The chromatographic signal was recorded by a Waters 745B integrator. The chromatogram and the chromatographic conditions developed by us [1] were shown in Fig.2.

RESULTS

To compare the HPLC method with the colorimetric method, 24 urine samples from unselected sheep were measured by both

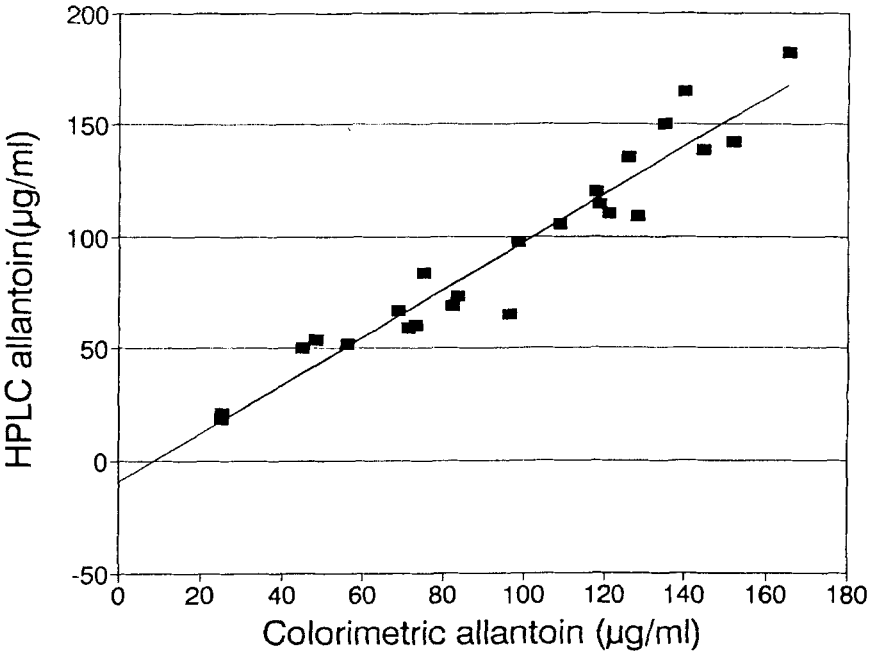

Fig 3.- Comparison of allantoin measurements of 24 sheep urine samples by HPLC and colorimetric methods.

techniques. Results are compared by using product-moment correlation (r) as a measure. In Fig.3, usual plot of results of HPLC method versus results of colorimetric method is shown. Correlation is good ($r = 0.962$) in a concentration range of 20 to 200 µg/ml.

According to Lee [10] we also used the interclass correlation as a better measure of method interchangeability. Lee suggests that two methods for measuring a quantitative variable can be judged interchangeable when the following conditions are met: first, the methods must not exhibit marked systematic bias. In Fig.4 the difference of readings between the two methods against the mean of the two methods is plotted. It could be seen that there is no marked systematic

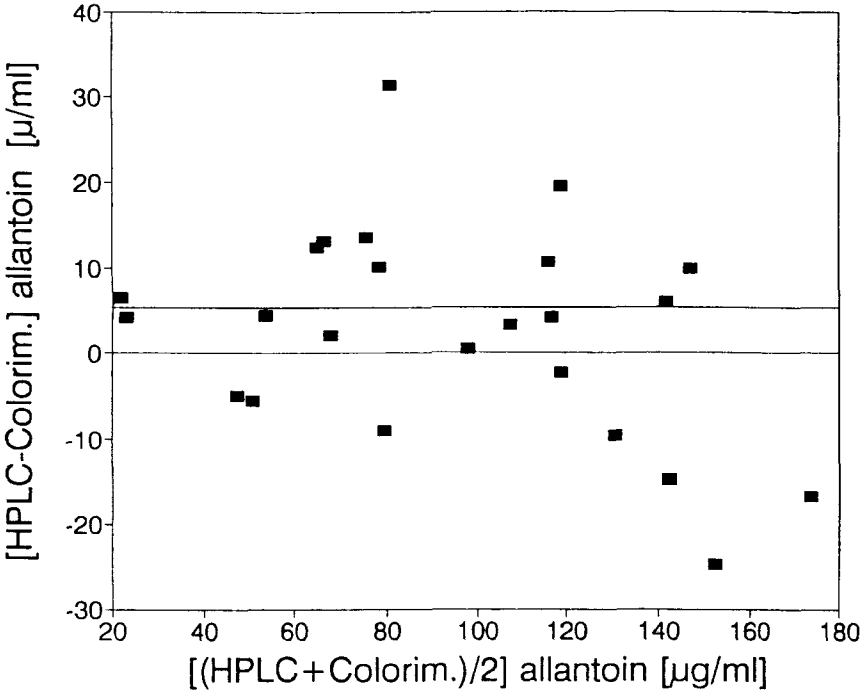

Fig 4.- Relationship between difference [HPLC - Colorimetric] and mean [(HPLC + Colorimetric)/2] assays of allantoin in 24 sheep urine samples obtained by HPLC and Colorimetric methods.

bias; second, the difference between the two mean readings is not statistically significant. Statistical analysis by the paired t-test ($t = 1.06$, $p = 0.29$) showed that the mean difference is not statistically significant; third, the lower limit of the 95% confidence interval of the interclass correlation is at least 0.75. The results of our comparison ($r_1 = 0.957$, lower limit for $r_1 = 0.916$) confirmed the agreement between these two methods. The methods are therefore interchangeable.

In summary it could be concluded that the RP-HPLC method [1] is a good substitute for the traditional colorimetric assay [2] for allantoin.

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