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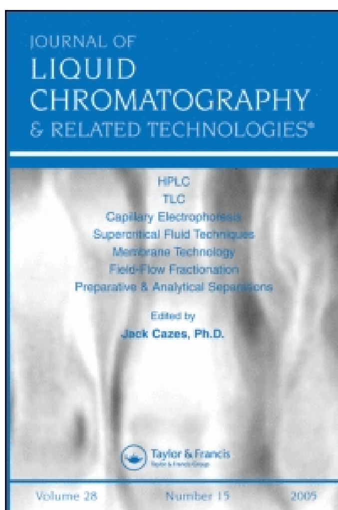

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HPLC METHOD FOR THE EVALUATION OF BLOOD ACETALDEHYDE WITHOUT ETHANOL INTERFERENCE

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ABSTRACT

The evaluation of acetaldehyde blood levels is important in view of possible toxic effects in the acute and chronic alcohol intoxication. Artefactual formation of acetaldehyde and its binding to erythrocyte components are the main problems that scientists have faced with in the measurement of acetaldehyde blood levels. The results reported herein show that addition of butyraldehyde as internal standard to the blood immediately after withdrawal allows to obviate these inconveniences. Aldehydes converted into their 2,4-dinitrophenylhydrazones are then analyzed by HPLC. The mean value of acetaldehyde blood concentration measured by this method in 15 healthy subjects was 12.2 $\pm$ 1.3 µM. The increase of acetaldehyde concentration in rabbits after ethanol infusion is also shown.

INTRODUCTION

Acetaldehyde is found in traces in the organism as the product of reactions occurring in the intermedi-

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ary metabolism (1). Significant concentrations are only found after ethanol ingestion.

Due to its chemical reactivity, acetaldehyde can be involved in manifestations of acute and chronic alcohol intoxication: so, it has been associated with the pathogenesis of alcoholic liver disease (2) and ethanol induced bone-marrow toxicity (3) and it was considered a basis of alcohol addiction (4). The research on acetaldehyde toxic effects has been prevented by problems connected with its determination in biological samples. To this regard, several studies were carried out concerning the measurement of acetaldehyde blood levels; the results obtained are discussed in details in a review article (5). Essentially, acetaldehyde concentration in blood can be either underestimated or overestimated: low values may be the result of an interaction of aldehyde with the erythrocyte proteins, as shown in rats for the binding with hemoglobin (6), or they may derive from a rapid metabolism of the compound possibly catalyzed by enzymes (7,8). To obviate this inconvenience, advice has been given (5) for a blood deproteinization to be performed within few seconds after blood withdrawal.However, just in the course of denaturation an artefactual acetaldehyde formation due to oxidation of the ethanol present in blood, has been observed, resulting in overestimation of blood content (9,10).

Some methods were proposed to obviate artefactual acetaldehyde formation; the use of a rapid denaturation by perchloric acid in order to avoid ethanol biological

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oxidation has been shown to be most reliable (5): in this case, reference curves should be prepared using control blood ethanol (9,10). This method however does not allow to know blood acetaldehyde basal concentration but only its increasing induced by alcohol consumption.

A rapid separation of plasma from blood can be carried out and followed by plasma deproteinization in which ethanol oxidation is no longer active. Plasma separation can be performed after addition to the blood of semicarbazide which traps acetaldehyde and avoids its binding to erythrocyte proteins (11,12). However, under the suggested conditions, the reagent seems not or hardly react with erythrocyte bound acetaldehyde.

It has been recently observed that if extration is carried out by an organic solvent on the whole blood added with 2,4-dinitrophenylhydrazine, high levels of acetaldehyde are found (13). In the present work we describe a method for the evaluation of acetaldehyde blood levels by HPLC after formation of its 2,4-dinitrophenylhydrazone in analogy with Thomas et al. (13). Addition to blood of butyric aldehyde as internal standard allows to obtain results corrected both for interaction of the aldehyde group of acetaldehyde with amino groups, and for formation and extraction of the derivative compound. Alcohol addition to blood does not modify the obtained results. The mean value of acetaldehyde blood levels measured by this method in 15 control subjects was 12.2 $\pm$ 1.3 µM (SEM).

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MATERIALS AND METHODS

Acetic and butyric aldehydes were purchased from Merck (Darmstadt, FRG); 2,4-dinitrophenylhydrazine was from C. Erba (Milan, Italy); isooctane was from Fluka (Buchs, Switzerland) and CH_3CN used in HPLC analysis from Merck; 2,4-dinitrophenylhydrazones(2,4-DNP) of acetaldehyde, acetone, propionaldehyde and butyraldehyde to be used as reference standards were prepared by the usual procedure (14). HPLC conditions were similar to those described by Selim (15). A stainless steel µ-Bondapack C_{18} (Waters Assoc., Milford, Mass.) 10 µ, 3.9 mm x 30 cm, reverse phase column was used. The derivatives were eluted from the column in a Waters 6000A solvent delivery system with 3 ml/min flow of $CH_3CN:H_2O$ (50:50, v:v). A UV detector (Waters, Mod.450) was set at 336 nm for the detection of the aldehyde derivatives. Under these conditions, retention times of the 2,4-DNP of acetaldehyde, acetone and butyraldehyde were 3'40", 4'50" and 7'40", respectively. Generally, butyraldehyde (100 nmol) in isopropanol (10 µl) was added as internal standard to 2 ml of heparinized blood, immediately after withdrawal. Plasma obtained by centrifugation at 4°C was treated with 3 M perchloric acid (0.7 ml), 2,4-dinitrophenylhydrazine in 6 N HCl (2.3 pmol, 100 µl), and 3 M sodium acetate (1.7 ml). Centrifugation at 15000 x g for 15 min gave a supernatant which was extracted by shaking with 2 ml of isooctane for 20 min. The organic phase was separated and the solvent evaporated to dryness. The residue was then dissolved in $CH_3CN:H_2O$ (50:50, v:v) and analyzed

by HPLC as described above. Blood used for measurement of acetaldehyde basal levels and for calibration curves was collected from healthy volunteers who had not consumed alcohol for at least 24 hrs, and it was immediately transferred to ice cold tubes.

The same procedure was used for blood collection from male New Zealand albino rabbits (2.5-2.8 kg b.w.) before and after treatment with 1.5 g/kg of ethanol. The dose was given by a 15-min infusion of a 50% aqueous solution at 0.7 ml/min into the ear vein.

Calibration curves were prepared adding butyraldehyde in isopropanol (100 nmol, 10 µl), and acetaldehyde as aqueous solution (10, 16, 20, 40 and 100 nmol) to blood aliquots (2 ml). The samples were then processed as described above for the measurement of blood levels. A curve was also prepared by mixing the 2,4-DNP of butyraldehyde (100 nmol) with 10, 16, 20, 40 and 80 nmol of the 2,4-DNP of acetaldehyde.

Levels of acetaldehyde in blood were calculated from the ratio of the peak areas of acetaldehyde and butyraldehyde 2,4-DNP on the basis of the calibration curve obtained in blood (Fig. 1). In some cases the level was calculated from the intercept of the calibration curve obtained using aliquots of the same blood as that of the sample.

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RESULTS

Fig. 1 shows the results obtained form the analysis of a typical calibration curve prepared by adding a constant amount of butyraldehyde and increasing amounts of

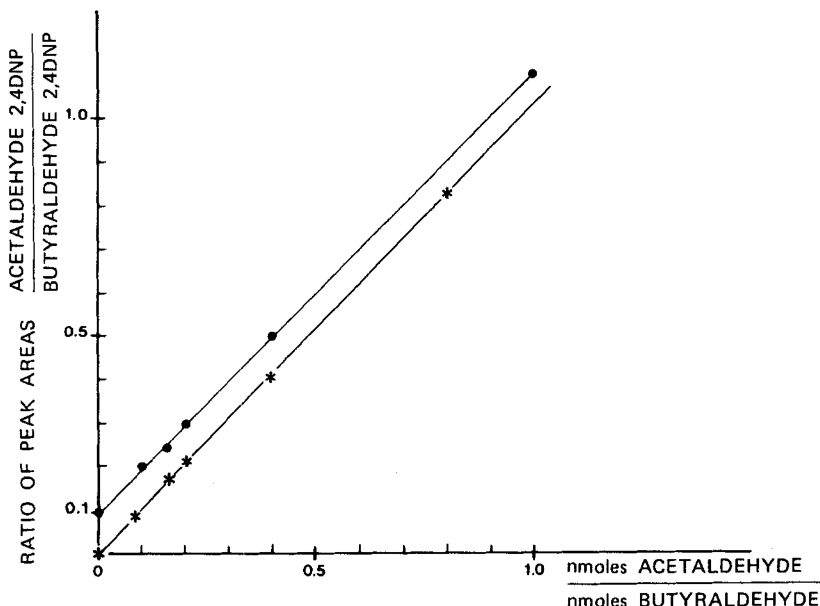

FIGURE 1

Linearity of the evaluation of blood acetaldehyde. Points represent results obtained in the analysis of duplicate samples: ●A constant amount of butyraldehyde (100 nmol) and increasing amounts of acetaldehyde were added to 2 ml of blood to obtain the ratios shown in the abscissa. Samples were then processed as described in the text to obtain 2,4-DNPs which were then analyzed by HPLC. *A constant amount of authentic butyraldehyde 2,4-DNP (100 nmol) and increasing amount of authentic 2,4-DNP of acetaldehyde were mixed to obtain the shown ratios. The compounds were dissolved in isooctane and analyzed by HPLC as described in the text.
Lines obtained by regression analysis were:
$y = 1.00(\pm 0.01)\ x + 0.1(\pm 0.005)$ for blood analysis
$y = 1.03(\pm 0.01)\ x + 0.002(\pm 0.005)$ for standard 2,4-DNP.
The intercept on the axis was significantly different from zero (t test) in the curve of blood analysis (●), whereas the line passed through zero in the analysis of authentic 2,4-DNP (*).
Comparison of the two lines (16) showed that they are parallel.

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acetaldehyde to human blood. When the ratio between nmoles of acetaldehyde and butyraldehyde added to the blood was plotted against the ratio between the peak areas of acetaldehyde and butyraldehyde 2,4-DNP, a line was obtained with a slope that did not significantly differ from that obtained when mixtures of the 2,4-DNP of acetaldehyde and butyraldehyde were directly analyzed by HPLC (Fig. 1). This ensured that if interaction of acetaldehyde with erythrocytes occurs, it is active to the same extent as with the butyraldehyde used as internal standard. While the line obtained from analysis of authentic 2,4-DNP passed through zero (Fig. 1), from the intercept observed in the analysis of the curve in blood a basal concentration of 5.0 $\pm$ 0.25 µM acetaldehyde was calculated. This value did not significantly differ from that obtained in the triplicate analysis of the same blood to which only butyraldehyde had been added as internal standard (5.0 $\pm$ 0.40 µM). The comparison of peak areas of the 2,4-DNP in the blood extracts with those in the authentic standards allowed to calculate a recovery from the blood of 31 $\pm$ 6.6% (mean $\pm$ SEM in 7 samples) for acetaldehyde at all tested concentrations and of 27 $\pm$ 3.2% for butyraldehyde. The results of analysis of the samples where 2,4-DNPs of acetaldehyde were obtained from aqueous solutions of the two aldehydes showed that the yield of the derivative formation and of its extraction was 44.3 $\pm$ 5.6% and 47 $\pm$ 2.7% for acetaldehyde and butyraldehyde, respectively. The lower recovery from blood seems to confirm that reaction occurs between the aldehydes and blood components. Basal blood levels of acet-

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TABLE 1

Blood Acetaldehyde Basal Levels in Healthy Subjects

Sex	Subject	Age	Acetaldehyde µM A	Acetaldehyde µM B
F	C.G.	30	11.2	-
	D.B.	20	9.5	-
	C.P.	33	9.1	-
	E.M.	24	6.8	-
	C.S.	26	8.6	9.2
	G.I.	60	19.7	21.6
	P.V.	35	8.6	9.0
	M.G.	33	13.2	11.2
M	R.S.	28	8.3	-
	A.R.	33	9.5	-
	G.M.	29	9.7	-
	F.B.	25	10.2	-
	L.B.	28	19.4	19.4
	M.P.	30	17.5	16.8
	A.F.	77	21.7	20.5

A = Values obtained from the analysis of duplicate aliquots of the same blood to which only butyraldehyde had been added.

B = Values extrapolated from the standard curve obtained by addition of a constant amount of butyraldehyde and increasing amounts of acetaldehyde to blood aliquots.

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aldehyde in 15 healthy subjects who had not consumed alcohol for at least 48 hrs are reported in Table 1. In no case a difference was observed between the level calculated from the intercept of the curve prepared for each blood sample as described in Fig. 1 and that deriving from the duplicate analysis of the same blood to which no acetaldehyde had been added.

Table 2 shows the results obtained from human blood mixed with ethanol. Direct blood denaturation induced an acetaldehyde level increase as already de-

TABLE 2

Acetaldehyde Levels in Human Blood containing Ethanol

Ethanol added to blood (µmol)	Acetaldehyde (nmol)	
	Blood denaturation	Plasma denaturation
0	108	104
30	255	104
60	305	103
120	480	114

Aliquots of blood taken from a healthy subject who had not consumed alcohol for at least 48 hrs were mixed with the shown amounts of ethanol. Denaturation was carried out with perchloric acid added either directly to the blood or to plasma after its separation. Butyraldehyde as internal standard was always added to blood before any other treatment. Results represent the mean of duplicate analyses.

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scribed (9,10), whereas by the procedure here reported no influence of ethanol was observed in the evaluation of aldehyde levels.

Fig. 2 shows the blood levels of both ethanol measured as described by Bücker and Redetzky (17) and acetaldehyde determined using the method reported here in rabbits treated with 1.5 g/kg ethanol. At the end of the 15-min infusion, both alcohol and acetaldehyde levels were significantly higher than basal levels. Ethanol levels then decreased as shown in Fig. 2, whereas a slight increase was observed for acetaldehyde. This further demonstrated that no aldehyde was artefactually produced from ethanol.

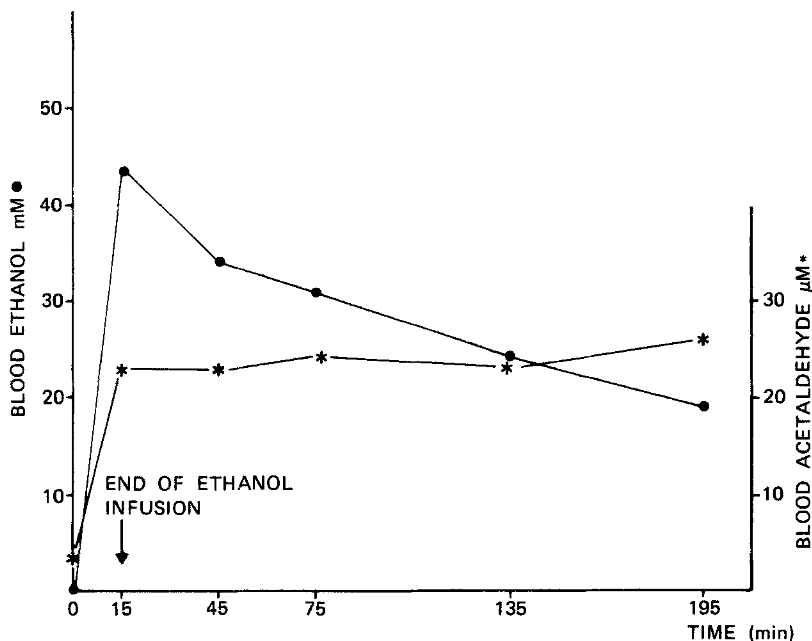

FIGURE 2

Ethanol concentration in blood was determined by the method of Bücker et al. (17). Points represent the mean of ethanol and acetaldehyde levels in two rabbits before (t_0) and after a 15-min infusion with 1.5 g/kg of ethanol. Assays were made in duplicate.

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DISCUSSION

The method here reported for evaluation of acetaldehyde blood levels is based on the transformation of the compound into its 2,4-DNP and on the derivative analysis by HPLC. In this regard, it does not differ from the method briefly described by Thomas et al. (13). These Authors actually suggest the addition of the reactive 2,4-dinitrophenylhydrazine directly into the blood, followed by extraction with a solvent they do

not specify. The determination by HPLC is made after addition to the extract of the 2,4-DNP of propionic aldehyde. We modified both internal standard and general procedure for the extraction. Choice of butyraldehyde as internal reference instead of propionaldehyde has been made because the 2,4-DNP of the latter showed a retention time very similar to that of the 2,4-DNP of acetone, under the analysis conditions reported in the present study and under others preliminarly experimented. As acetone resulted to be always present as a contaminant in the assayed extracts, it was not possible to calculate the peak area of the propionic aldehyde derivative correctly. The longer retention time of butyric aldehyde derivative allowed instead a correct evaluation of the peak area. Moreover, in our experimentation, the addition of solvents directly into blood as suggested by Thomas et al. (13) generated gels from which the organic phase was hardly separable. There was, therefore, the necessity for a denaturation with perchloric acid but if made directly in blood when it contained alcohol one could have noted the production of artefactual acetaldehyde in a quantity depending on alcohol concentration. This did not occur when denaturation was made after plasma separation.

On the other hand, since recovery of the 2,4-DNP of the acetaldehyde added to blood was constantly lower than that obtained by addition of the acetaldehyde to water, the compound disappearance may be reasonably due to interaction with the erythrocyte proteins (6). Assuming that the interaction is not specific for acet-

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aldehyde but depends only on the aldehyde group reactivity, we added butyric aldehyde as internal reference directly to the blood. The recovery of the derivative of this aldehyde corresponds to that obtained for acetaldehyde at all the tested concentrations; hence the linear response reported in our results. A good reproducibility was found; indeed, the acetaldehyde concentration values observed in two aliquots of the same blood specimen did not differ from one another more than 10% for all the examined blood samples.

The results of the analysis of basal levels in control subjects showed a concentration of 12.2 $\pm$ 1.3 µM. This value is remarkably higher than those obtained with the method implying addition of semicarbazide to blood (11,12). This may be explained by the fact that at least part of blood acetaldehyde remains bound to macromolecules even in the presence of excess semicarbazide. Demonstration of this is also given by the partial recovery of 2,4-DNP both of acetic and butyric aldehydes added to blood.

Our values, however, are lower than that found by Thomas et al. (13) in control subjects, which was 60 $\pm$ 18 µM. Yet, such a difference might depend on special dietary conditions of the different ethnic groups to which subjects belong.

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