Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 7, pp. 701–706, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

Enzymatic determination of ethanol in saliva by flow injection analysis

P. LINARES, J. RUZ, M. D. LUQUE DE CASTRO and M. VALCARCEL

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, Córdoba, Spain

Abstract: Enzymatic photometric and fluorimetric methods are proposed for the determination of ethanol in saliva with determinative ranges of 2.5–15.0 μg ml^{-1} and 1.0–20.0 μg ml^{-1} respectively, i.e. covering the legal ranges after 1 : 100 dilution. A comparison between the ethanol content in saliva, breath and blood has been carried out on 12 individuals; the determination in saliva has been shown to be a good option.

Keywords: *Flow injection analysis; ethanol measurement; saliva.*

Introduction

Ethanol is one of the most widespread and socially accepted "drugs" and is responsible for a high percentage of traffic accidents. Legislation covering alcohol intake by drivers is often strict although enforcement varies greatly between countries. Its intake is monitored by measuring the concentration in various biological fluids (blood, urine, saliva or breath), but generally expression is in terms of blood content, derived when necessary by correlation from levels found in other body media.

Monitoring of ethanol in drivers by road side police is effected through the content in breath [1, 2] which may be readily obtained by a non-skilled worker. Blood measurements entail venepuncture which may only be performed by skilled staff and though blood analysis is more accurate than breath measurement [3], it is also more laborious [2, 3]. As alternatives of intermediate complexity, determination in urine [4–7] — regarded as rather error-prone by some authors [8] — and in saliva [9–11] have been proposed, though saliva has been scarcely investigated. A device designed for the analysis of ethanol in saliva — the Alcolmeter AE-dl [10] — employs an electrochemical fuel cell to sense and measure the concentration of ethanol in the sealed head-space vapour above the fluid sample. The fuel cell is housed in a "sensor head" and is an integral part of the sample aspirating system which automatically introduces a fixed volume (1 ml) into the head-space of the detector. The alcohol in the sample is captured by the platinum electrode in the fuel cell and electrochemically oxidized to acetic acid. The reaction releases electrons from the alcohol molecule, producing an electron flow which gives a voltage change across an external resistance. This is directly proportional to the concentration of ethanol in the head-space vapour, which in agreement with

Henry's Law, is in equilibrium with the fluid alcohol concentration. Recently, Girotti *et al.* [11] proposed a very sensitive bioluminiscent method for the determination of ethanol in serum and saliva, making use of a very complex chemical system based on the reaction of the analyte with NAD(P)H: flavin mononucleotide oxidoreductase and bacterial luciferase co-immobilized on a nylon coil, and alcohol dehydrogenase separately immobilized on a second nylon coil. The system involves flow with air-segmentation and has a measurement range between 500 and 2500 pmol of ethanol. The results obtained in both biological fluids have not been correlated or applied to real samples in a systematic way.

In this paper we propose a simple method for the determination of ethanol in saliva by flow injection analysis (FIA) [12] based on the oxidation of the analyte to formaldehyde by NAD^+ in the presence of alcohol dehydrogenase. The development of the reaction is monitored photometrically or fluorimetrically through the reduced form of the coenzyme (NADH). The method has been tested on the saliva of several male and female individuals previously given a measured amount of ethanol. Samples were taken at fixed times after ingestion. The results have been correlated with those found in blood and breath samples and assayed simultaneously.

Experimental

Apparatus

The following instruments were used. Perkin–Elmer Lambda 1 spectrophotometer equipped with a Hellma 178.12QS flow-cell (inner volume 18 μl); Perkin–Elmer LS-1 LC fluorescence detector with a 1.5 cm square flow-cell; Radiometer REC 80 recorder; Gilson Minipuls-2 peristaltic pump; Tecator FIA 5020 analyser; a "home made" dual injection valve with variable injection volume; Tecator T-III chemifold; Selecta S-382 thermostat; Drager Alcotest, mod. 7310.

Reagents

Stock aqueous solution of potassium pyrophosphate (1.0 g l^{-1}). Carrier: aqueous solution of potassium pyrophosphate (0.075 M) with 8 g l^{-1} semicarbazide hydrochloride, adjusted to pH 9.0 with sodium hydroxide. Reagent solution containing 9000 U of alcohol dehydrogenase (ADH) and 45 mg NAD^+ (Boehringer Mannheim), diluted to 100 ml with potassium pyrophosphate buffer pH 9.0.

Sample preparation

Fifty microlitres of saliva are placed in a 5-ml volumetric flask and made up to volume with potassium pyrophosphate buffer pH 9.0.

Manifold

The FIA configuration used is shown in Fig. 1. A dual injection valve allows the simultaneous insertion of sample and enzyme–coenzyme solution in two buffer channels, both with identical geometrical and hydrodynamic characteristics. They merge at a point and the mixture is then transported to the flow-cell where flow is halted by stopping the peristaltic pump in synchronism with the injection valve; reaction development is monitored for a preselected time.

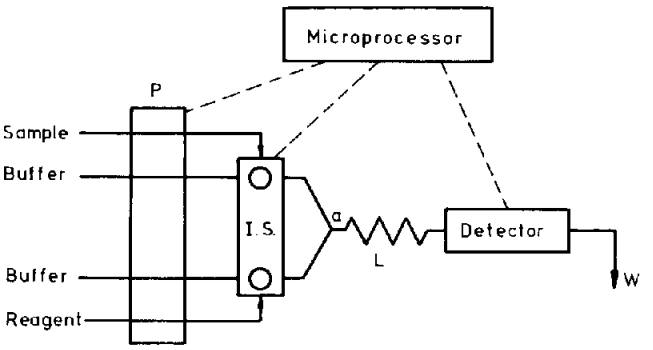

Figure 1
FIA scheme for determination of ethanol in saliva with photometric and fluorimetric detection. For explanation see text.

Results and Discussion

The FIA configuration (Fig. 1) has two aims: (1) small reagent consumption (as much as sample) resulting from the use of the merging zones mode [13, 14], which reduces the enzyme and coenzyme expenditure to a minimum; (2) elimination of the signal from the sample matrix by making kinetic measurements (stopped-flow technique). Two different types of optical detector have been compared: photometric (λ_{max} = 340 nm) and fluorimetric (λ_{ex} = 340 nm, λ_{em} = 460 nm).

Optimization of variables

The optimum FIA, chemical and physicochemical variables influencing the system were obtained by the univariate method and the results are similar to those obtained for methods for the determination of ethanol in blood with the same chemical system described previously [15, 16]. A short reactor length (20 cm) required for the sample-reagent plug to arrive at the detector immediately after mixing has been achieved so that for the higher rates, the reaction can be monitored in its early stages. This requirement was also a major consideration in the selection of an optimum flow-rate of 1.8 ml min^{-1} (slower flow-rates delay the arrival of the reactant plug at the cell, while faster ones result in unreproducible measurements because the plug has not taken on a definite geometrical shape before it reaches the detector). The analytical signal increases with the injection volume, so a value of 100 µl was chosen as a compromise between sensitivity of sample and reagent consumption. The optimum concentration of enzyme (90 U ml^{-1}) and coenzyme (0.45 mg ml^{-1}) was such as to give negligible signal changes with concentration. Semicarbazide was used to trap the acetaldehyde formed and exerted its maximum effect at a concentration of 8 g l^{-1} at pH 9.0. Reagents and FIA system were monitored at 25°C in a thermostatically controlled bath, though the signal change over the temperature range studied (15–45°C) was very small. The optimum delay and stop times for the stopped-flow technique were 11 and 30 s respectively. It is not possible to use immobilized enzymes to reduce analytical costs because of the need to eliminate the sample matrix signal by performing kinetic measurements.

Determination of ethanol in saliva

Both methods, photometric and fluorimetric, have been tested with different dilutions of saliva spiked with ethanol. The photometric method gives poor precision for dilutions

below 1 : 50, but using fluorimetry 1 : 10 dilutions are possible. In both cases the lower limit of detection is better than for techniques applicable to blood [15, 16], where the concentration range is similar.

Calibration curves produced with saliva samples spiked with ethanol and made up to volume with potassium pyrophosphate buffer (dilution 1 : 100) are shown in Table 1. It will be seen that fluorimetry is better than photometry in: (a) range (1.0–20.0 μg ml^{-1} versus 2.5–15.0 μg ml^{-1}); (b) lower limit of determination (0.26 μg ml^{-1} versus 0.47 μg ml^{-1}) and (c) reproducibility (r.s.d. 0.50% versus 0.68%); the linear correlation is similar (r^2 = 0.9999 and 0.9998 for the fluorimetric and photometric methods respectively). The sample frequency is the same for both (40 h^{-1}).

Comparison of the ethanol content of saliva, blood and breath

To carry out this study, 12 individuals (6 males and 6 females) were given 150 and 100 ml of whisky (42°) 2 h after a light breakfast. The three samples from each person were taken almost simultaneously 45 min after ingestion, a time previously shown to give maximum concentration in the different fluids.

The concentration in breath was measured by Draeger Alcotest, the concentration in blood by a fluorimetric method using the enzyme immobilized on controlled pore-glass [17]; 50 μl of blood were diluted to 5 ml with the carrier. The concentration in saliva was determined by the kinetic photometric method proposed in this paper.

Figure 2 shows in part a, the correlation of the results in saliva and blood; and in part b the correlation between results in saliva and breath. Table 2 compares the analyte concentrations in the three fluids through the relation of each method to itself [18] and

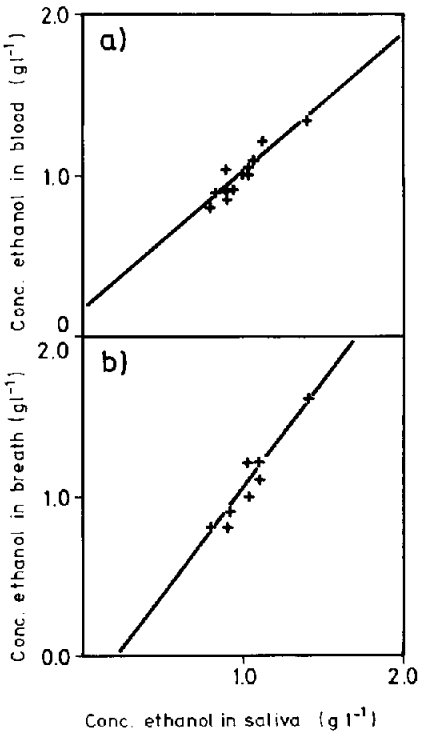

Figure 2
Correlation between the ethanol concentration in saliva and that found in (a) blood; (b) breath.

Table 1
Features of the methods

Method	Equation*	Regression coefficient	Determination range†	Determination limit %	r.s.d.‡	Sampling frequency (h^{-1})
Photometric	$\Delta A = 0.0157.0.0084\|EtOH\|$	0.9998	2.5–15.0	0.47	0.68	40
Fluorimetric	$\Delta I_f = 14.65 + 12.57\|EtOH\|$	0.9999	1.0–20.0	0.26	0.50	40

*ΔA = absorbance increment; ΔI_f = fluorescence intensity increment.
†$\mu g\ ml^{-1}$.
‡for 11 samples injected in triplicate, 5 $\mu g\ ml^{-1}$.

Table 2
Comparison between ethanol concentration in saliva with that found in the breath and blood of 12 individuals 45 minutes after drinking whisky

People	Saliva	Breath*	Blood*	Conc. saliva/ conc. $saliva_1$	Conc. breath/ conc. $breath_1$	Conc. blood/ conc. $blood_1$	Conc. saliva/ conc. breath	Conc. saliva/ conc. blood
1	0.79	0.8	0.81	1.00	1.00	1.00	1.00	1.00
2	1.40	1.6	1.33	1.77	2.00	1.64	0.89	1.08
3	0.83	0.8	0.89	1.05	1.00	1.10	1.05	0.95
4	0.90	0.8	0.90	1.14	1.00	1.11	1.14	1.03
5	0.90	0.8	0.80	1.14	1.00	1.06	1.14	1.07
6	1.02	1.2	1.03	1.29	1.50	1.27	0.86	1.01
7	0.81	0.8	0.89	1.03	1.00	1.10	1.03	0.94
8	1.02	0.9	1.00	1.29	1.12	1.23	1.15	0.95
9	1.11	1.1	1.12	1.40	1.37	1.38	1.02	0.99
10	1.10	1.2	1.06	1.39	1.50	1.31	0.93	0.94
11	0.92	0.9	0.90	1.16	1.12	1.11	1.03	0.96
12	1.03	1.0	1.05	1.30	1.25	1.29	1.04	1.01

*Concentration in $g\ l^{-1}$.

between methods, thereby eliminating instrumental errors. There is good agreement between the ethanol concentrations in blood and saliva, and slightly worse agreement between those of saliva and breath samples. This might be attributable to the lower precision of breath measurements. The correlation equations are: $y = -0.357 + 1.368x$ and $z = 0.170 + 0.828x$ where x denotes the saliva concentration and y, z those found in blood and breath, respectively, expressed in g l^{-1}.

It therefore appears that measurement in saliva is a good alternative to measurement in breath which is more prone to error. The determination in blood requires drawing a sample which is not always possible. Saliva measurements can be of value whenever the determination of ethanol in breath is suspected.

Acknowledgement: This work has been supported by a grant from "Jefatura Central de Tráfico del Ministerio del Interior de España", Spain.

References

[1] J. Ruz, A. Fernández, M. D. Luque de Castro and M. Valcárcel, *J. Pharm. Biomed.* **4** (5), 545–558 (1986).
[2] *Ibid* **4** (5), 559–564 (1986).
[3] M. F. Mason and K. M. Dubowski, *J. Forensic Sci.* 9–41 (1975).
[4] W. Froentjes, *Chem. Weekbl.* **59**, 369–377 (1963).
[5] P. Payne, D. Foster, W. Hill and D. Wood, *Br. Med. J.* **3**, 819–823 (1967).
[6] N. Jain and R. Gravey, *J. Chromatogr. Sc.* **10**, 257–262 (1972).
[7] *Ibid* **12**, 209–218 (1974).
[8] W. Morgan, *J. Forens. Sci. Soc.* **5**, 15–21 (1965).
[9] K. Feller and G. le Petit, *Int. J. Clin. Pharmac.* **15**, 468–469 (1977).
[10] T. P. Jones and P. M. Williams, *Ass. Forensic Toxicologists*, 86–94 (1979).
[11] S. Girotti, A. Roda, S. Ghini and L. Piacentini, *Anal. Chim. Acta* **183**, 187–196 (1986).
[12] M. Valcárcel and M. D. Luque de Castro, in *Flow Injection Analysis: Principles and Applications*. Ellis Horwood, Chichester, 1987.
[13] H. Bergamin Fo., E. A. G. Zagatto, F. J. Krug and B. Reis, *Anal. Chim. Acta* **101**, 17–35 (1978).
[14] E. A. G. Zagatto, F. J. Krug, F. Bergamin Fo., S. S. Jorgensen and B. Reis, *Anal. Chim. Acta* **104**, 279–285 (1979).
[15] A. Fernández, J. Ruz, M. D. Luque de Castro and M. Valcárcel, *Clin. Chim. Acta* **148**, 131–137 (1985).
[16] J. Ruz, A. Fernández, M. D. Luque de Castro and M. Valcárcel, *Quím. Clín.* **4** (3), 171–175 (1985).
[17] J. Ruz, M. D. Luque de Castro and M. Valcárcel, *Analyst* (in press).
[18] Y. Sahlestrom, S. Twengström and B. Carlberg, *Anal. Chim. Acta* **187**, 339–342 (1987).

[*Received for review 19 March 1987*]