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DETERMINATION OF FREE ACETALDEHYDE IN BLOOD AS THE DINITROPHENYLHYDRAZONE DERIVATIVE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and sensitive method is proposed for the measurement of acetaldehyde in human blood. Venous blood samples were collected in EDTA Vacutainer tubes, and treated immediately with 0.6 *M* ice-cold perchloric acid in saline. After centrifugation at 4°C, the supernatants were treated with dinitrophenylhydrazine reagent. After addition of the internal standard (crotonaldehyde dinitrophenylhydrazone) and 3 *M* sodium acetate, the derivatives were extracted and analysed by high-performance liquid chromatography (HPLC) using an Ultrasphere ODS column. The compounds were separated using acetonitrile—water as the mobile phase and detected at 356 nm. A blank determination was carried out for each analysis and subtracted from the results. The specificity of the method was tested by UV and mass spectrometry and the purity of the derivatives by capillary gas chromatography. The recovery of blood acetaldehyde was 98%. Interference from ethanol was minimized by using the tripotassium salt of EDTA as an anticoagulant. The sensitivity of the method can be increased dramatically using microbore HPLC. The level of acetaldehyde was found to be 0.41 ± 0.13 μM (mean ± S.D.) for eight fasting controls and 0.91 ± 0.73 μM for fourteen alcoholics ($p<0.05$). At 30 min after oral administration of ethanol (0.8 g/kg), the ethanol levels were 16.3 ± 2.8 and 17.7 ± 2.5 mM and the acetaldehyde levels were 1.67 ± 0.35 and 3.13 ± 2.43 μM ($p<0.05$) for the controls and alcoholics, respectively.

INTRODUCTION

The measurement of acetaldehyde, the first metabolite of ethanol in blood, is of a great interest. This highly reactive compound can form adducts with proteins [1] and it has been implicated in the pathogenesis of alcoholism [2]. Its determination in blood is difficult, as demonstrated by the discrepancies in the levels measured by several workers, reported by Eriksson [3]. There are two possible reasons: first, acetaldehyde binds reversibly and irreversibly to proteins and/or is rapidly metabolized by enzymes [4]; conversely, artefac-

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tual acetaldehyde can be produced from the ethanol present in the sample during the deproteinization. Although many solutions have been proposed [5—7], this problem has not yet been overcome.

Most assays for acetaldehyde involve precipitation of blood proteins with an acid (perchloric acid), centrifugation and analysis by headspace gas chromatography (GC) [3]. The limit of quantification afforded by these procedures is about 1 μM. Eriksson [3] concluded that acetaldehyde should either be absent or present at nanomolar levels in the blood of normal subjects and it was therefore necessary to improve the sensitivity of the method. This can be achieved using high-performance liquid chromatography (HPLC). The free acetaldehyde was converted into its 2,4-dinitrophenylhydrazone (DNP) derivative and then quantified by HPLC with UV detection. Two methods based on this principle have recently been described. The first, proposed by Lynch et al. [8], is sensitive (detection limit 0.1 μM) but is time-consuming and not very convenient in the clinical laboratory because labelled formaldehyde is used as an internal standard. The method proposed by Pezzoli et al. [9] seems easier to carry out but overestimates the levels for healthy subjects (12.2 ± 1.3 μM). Moreover, the latter method is concerned with plasma acetaldehyde, which does not reflect total blood acetaldehyde.

The objective of this investigation was to develop a reliable, rapid and more sensitive HPLC method for the measurement of free acetaldehyde in blood.

EXPERIMENTAL

Reagents and standards

All solvents and chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, F.R.G.). The following reagents were used: 0.6 *M* perchloric acid in 0.15 *M* sodium chloride; 2,4-dinitrophenylhydrazine (1 mg/ml) in 6 *M* hydrochloric acid, prepared immediately prior to the analysis; acetonitrile, HPLC grade (Serlabo, France); 1.25 m*M* crotonaldehyde (DNP) in doubly distilled methanol. Aldehyde or ketone DNP standards were synthesized as described by Vogel [10].

Because of the low levels of acetaldehyde in blood, special precautions were necessary to minimize contamination by environmental aldehydes from cigarette smoke. Glassware was soaked overnight in chromic acid and washed with ultra-pure water.

Chromatography

The HPLC system consisted of a continuous constant-flow delivery module (Gilson, Model 302, F) equipped with a Rheodyne Model 7125 injection valve (20-μl loop) and a SpectroMonitor III variable-wavelength detector (LDC, Riviera Beach, FL, U.S.A.), operated at 356 nm at 0.1 a.u.f.s. A Shimadzu (Kyoto, Japan) C-R3A integrator recorder was used. The HPLC column (15 cm × 4.6 mm I D.) was packed with 5-μm Ultrasphere ODS. The chromatographic system was operated at room temperature. The mobile phase was acetonitrile—water (65:35, v/v). The solvents were filtered through a 0.45-μm membrane filter (Millipore, Milford, MA, U.S.A.). The flow rate was 1.25 ml/min and the pressure was 245 bar.

For microbore chromatography, the micro-HPLC system consisted of a Gilson Model 302 pump, modified for microanalysis, and a Shimadzu SPD-2A detector equipped with a 0.5 μl microcell. The column (250 mm × 1 mm I.D.) was packed with 10 μm Partisil ODS-3. The flow-rate of the mobile phase, acetonitrile—water (75:25, v/v), was 50 μl/min with a back-pressure of 40 bar.

The GC analysis was carried out on a glass capillary column (20 m × 0.32 mm I.D.) coated with OV-1 of film thickness 0.15 μm. Injection was carried out with a cold on-column injector. The oven temperature, being at 40°C during injection, was ballistically heated to 150°C and then programmed to 200°C at a rate of 5°C/min. The carrier gas was hydrogen at 0.5 bar. The gas chromatograph was a Model 2150 (Carlo Erba, Milan, Italy) equipped with a flame ionization detector.

Subject and blood specimens

Fourteen male alcoholic volunteers (mean age 42.5, range 23—57 years) seeking admission to an alcohol detoxification programme and eight healthy men (mean age 24.2, range 22—26 years) were investigated. The diagnosis of alcoholism was confirmed by evaluation of the γ-glutamyl transpeptidase activity in the serum (mean ± S.D. = 98 ± 112 I.U.; normal value < 25 I.U.) and by measuring the mean corpuscular volume (mean ± S.D. = 97 ± 6 μm^3, normal value < 87). The liver function, ascertained by serum protein electrophoresis and transaminase determination, was normal. Venous blood was collected in EDTA Vacutainer tubes before any medication. Ethanol and acetaldehyde concentrations were measured before and 30 min after an oral intake of ethanol [0.8 g/kg as a 16% (v/v) solution in dextrose].

Procedure

A 1-ml volume of blood was immediately mixed with 4 ml of ice-cold deproteinization reagent. After centrifugation (2000 *g* for 10 min at 4°C) the supernatant was transferred into an ice-cold tube and treated immediately or frozen and stored for a few days at −20°C. Then, 100 μl of the dinitrophenylhydrazine reagent, 2 ml of 3 *M* sodium acetate, 10 μl of internal standard and 2 ml of isooctane were added to 4 ml of the supernatant. After mechanical shaking for 20 min isooctane was separated and evaporated to dryness under a stream of nitrogen at 60°C. The residue was dissolved in 0.3 ml of acetonitrile—water (65:35, v/v) for HPLC analysis.

The relative response factor of acetaldehyde DNP and crotonaldehyde DNP (internal standard) was determined by analysing acetaldehyde standard solutions (0—40 μ*M*) according to the same procedure as for blood samples, i.e., using 1 ml of standard solution instead of 1 ml of blood. Peak-area measurements were used for quantification. For each analysis, the peak area of the blank was subtracted. The results are expressed in μ*M* acetaldehyde.

For the recovery experiments, acetaldehyde in aqueous solution (10 μl) was added to 1 ml of blood collected in tubes containing EDTA to give concentrations of 1, 2, 3, 5, 10, 20 and 40 μ*M*. The samples were immediately mixed with ice-cold perchloric acid and then processed as described above.

The artefactual formation of acetaldehyde from ethanol was studied. In

order to minimize the interference of ethanol in the measurement of acetaldehyde, blood was collected in tubes containing the tripotassium salt of EDTA (final concentration 5 m*M*). Moreover, Eriksson's recommendations [3] concerning the deproteinization conditions of the samples were followed.

RESULTS AND DISCUSSION

The use of an internal standard enhances the precison of the chromatographic method. [^{3}H]Formaldehyde or butyraldehyde has previously been reported as internal standards [8, 9]. The use of a labelled internal standard is not very practical and trace amounts of butyraldehyde were present in some reagents. We chose crotonaldehyde as it was not detected in blood (Fig. 1). Preliminary experiments showed that DNP derivatives are lipid-soluble and precipitate with lipoproteins during the deproteinization step. Hence the DNP reagent and internal standard must be added after the precipitation of proteins with perchloric acid. However, as the stock solution of crotonaldehyde was unstable, it was added as a secondary internal standard, i.e., added to the supernatant as the DNP derivative after the denaturation step.

Fig. 1A shows the chromatogram of a standard solution of aldehyde and ketone DNPs. Acetaldehyde and crotonaldehyde DNPs were completely resolved from the other compounds. Table I summarizes the retention data of the DNP derivatives on Ultrasphere ODS and Partisil ODS columns.

Fig. 1B shows typical chromatograms of a blood sample from a control

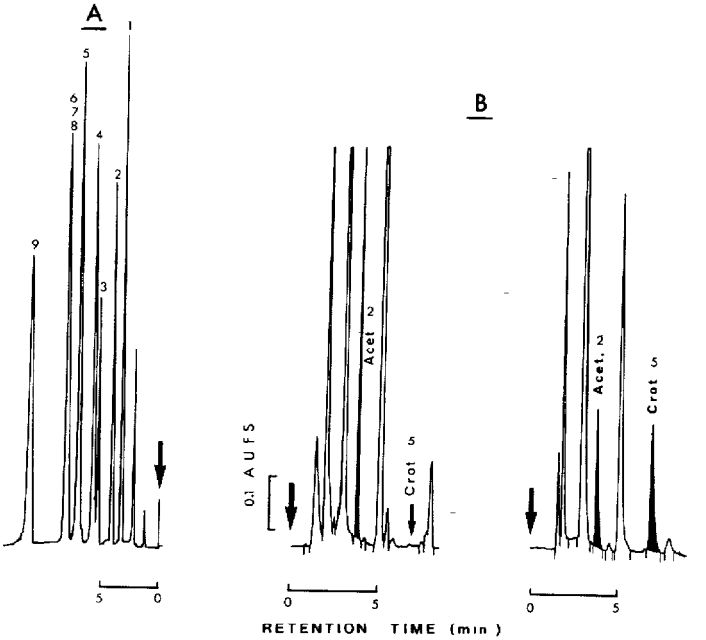

Fig. 1. (A) HPLC trace of a standard solution of aldehyde and ketone DNPs (see Table I for identification of compounds). (B) Chromatogram of blood sample without and with internal standard (crotonaldehyde DNP). Arrow = injection; Acet. = acetaldehyde DNP; Crot. = crotonaldehyde DNP.

TABLE I

CAPACITY OF FACTORS (k') FOR ALDEHYDE AND KETONE 2,4-DINITROPHENYLHYDRAZONES

Elution conditions: Ultrasphere ODS: acetonitrile—water (60:40, v/v); Partisil ODS: acetonitrile—water (75:25, v/v).

No.	Compound	k'	
		Ultrasphere ODS	Partisil ODS
1	Methanal	1.50	1.07
2	Ethanal (acetaldehyde)	2.16	1.40
3	Acetone	3.17	–
4	Propanal	3.50	1.92
5	*trans*-2-Butenal (crotonaldehyde)	4.50	2.29
6	Butanone	5.15	2.59
7	Butanal	5.55	–
8	Isobutanal	5.65	2.59
9	Pentanone	8.00	–

subject with and without internal standard. These chromatographic profiles illustrate the lack of interference of biological compounds with acetaldehyde or the internal standard.

Method validation

A linear relationship (r = 0.9999) between peak-area ratio (acetaldehyde DNP/crotonaldehyde DNP) and the concentrations of the aqueous solution of acetaldehyde (0—40 μM) was obtained. The linear regression equation was $y = 0.325x$ (±0.001) − 0.20 (±0.02).

The precision of the assay was determined for blood with and without added acetaldehyde (Table II). Coefficients of variation within and between assays were 6 and 10.3%, respectively, for a blood concentration of 1 μM acetaldehyde and 2.92 and 3.78% for 5 μM acetaldehyde.

The accuracy was tested by adding acetaldehyde to samples of blood in the concentration range 0—40 μM. The recovery was 88.5–101% and regression analysis showed a linear relationship between added and recovered acetal-

TABLE II

PRECISION OF THE ASSAY DETERMINED FOR BLOOD (a) WITHOUT AND (b) WITH ADDED ACETALDEHYDE (n = 10)

		x (μM)	Coefficient of variation (%)
Within-assay	(a)	1.14	6
	(b)	5.14	2.92
Between-assay	(a)	0.97	10.3
	(b)	5.13	3.78

dehyde: $y = 0.982x$ (±0.008) − 0.105 (±0.155) ($r = 0.9998$). This recovery is higher than those previously reported by Lynch et al. [8] and Pezzoli et al. [9] which were 85 and 31%, respectively.

The limit of quantification (L_q) is defined according to Curie [11] as the limit at which the procedure will be sufficiently precise to yield a statisfactory quantitative estimate of the unknown concentration. For a 10% precision, the determination limit is $L_q = 10\sigma_{blank}$, where σ_{blank} is the standard deviation of the measurement of acetaldehyde in the blank sample. L_q was evaluated as 0.46 μM for blood acetaldehyde. Further, a level of five times the signal-to-noise ratio may be used to estimate the detection limit in UV detection. This level was calculated to be 235 pg (5.34 pmol) of acetaldehyde under analytical HPLC conditions. The use of a microbore column dramatically enhances the detection limit. The minimum detectable mass is directly related to the square of the column radius [12]. This detection limit for acetaldehyde for the microbore colum was 15 pg (0.34 pmol) (Fig. 2). This level was lower than that measured by electrochemical detection [13].

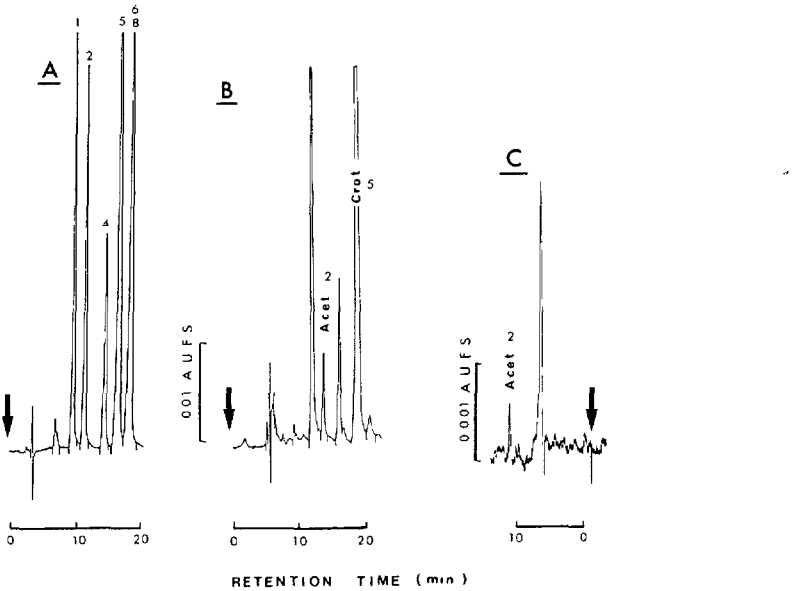

Fig. 2. Microbore HPLC trace. (A) Chromatogram of a standard solution of aldehyde and ketone DNPs (see Table I for identification of compounds); (B) chromatogram of a blood sample; (C) chromatogram obtained with 15 pg of acetaldehyde injected. Arrow = injection; Acet. = acetaldehyde DNP; Crot. = crotonaldehyde DNP.

Specificity

In order to ascertain the identity of the acetaldehyde HPLC peak, the UV spectra of acetaldehyde DNP and crotonaldehyde DNP eluted from the column were compared with those of the pure compounds and were found to be similar.

As acetaldehyde DNP can be determined by GC, the HPLC eluate was collected and analysed by capillary GC. As shown in Fig. 3, the retention

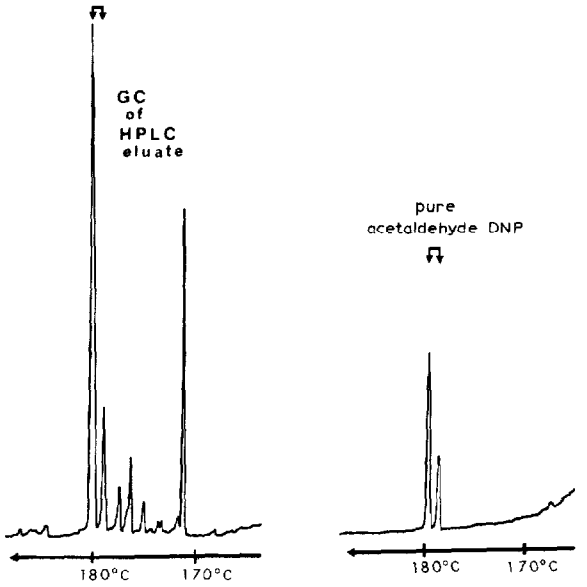

Fig. 3. Gas chromatogram of the HPLC eluate and pure acetaldehyde DNP on an OV-1 glass capillary column. For conditions, see text.

times were identical for blood and pure samples. Owing to the asymmetric nitrogen, geometric isomers of the *syn/anti* type were produced during DNP formation. Whereas this separation is usually considered as a disadvantage in quantitative work [14], it can be regarded here as an additional criterion for the identification of acetaldehyde DNP. The *syn/anti* ratio was not identical for the standard and the blood sample peaks because the reaction conditions were different. Conversely, crotonaldehyde DNP was not resolved into two peaks. This result is in agreement with previous work [14].

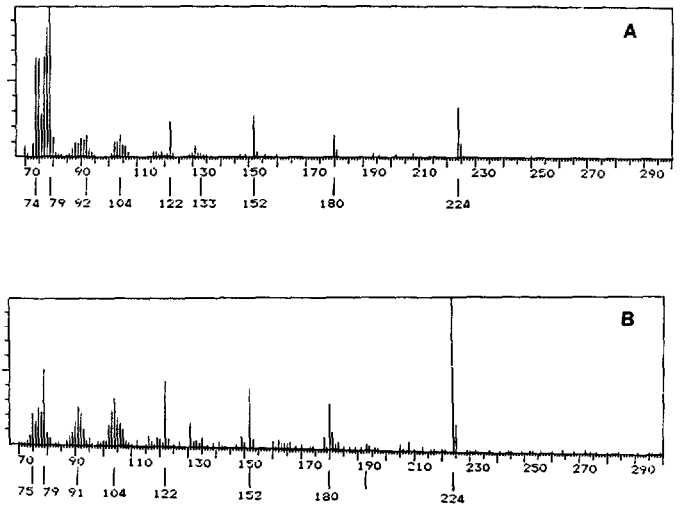

Fig. 4. EI mass spectra of (A) acetaldehyde DNP standard and (B) blood sample.

Moreover, the mass spectrum of acetaldehyde DNP standard was compared with that from blood eluted at the same retention time by HPLC (Fig. 4). Using the electron-impact (EI) ionization mode, the base peak was at *m/z* 224, which corresponds to the molecular ion peak. The ion at *m/z* 180 corresponded to the elimination of a CH_3CHO residue with rearrangement as previously reported [15]. The loss of water from fragment species in several dinitrophenylhydrazones has been described and explains the ion at *m/z* 152 [16]. Peaks at *m/z* 131 and 122 were also found for both samples.

Artefactual formation of acetaldehyde from ethanol

Research on the toxic effects of the acetaldehyde has been hindered by problems connected with its determination in blood. Artefactual acetaldehyde can be produced from ethanol present in the sample during deproteinization. The proposed mechanism involves the formation of a superoxide radical during the autoxidation of ascorbic acid [5] or the oxidation of oxyhaemoglobin to methaemoglobin. Sodium azide can minimize this artefact but is not convenient for routine use because of its toxicity. Deproteinization with dilute perchloric acid in saline [3] was reported to induce minimal acetaldehyde formation. As shown in Fig. 5, this artefact is much more important when heparin is used as an anticoagulant compared with K_3 EDTA. This powerful complexing agent may decrease the formation and/or the reactivity of superoxide radical, which explains the low artefactual acetaldehyde formation (0.8 μM for 17 mM ethanol). This artefact was not significantly different for alcoholics and controls (results not shown).

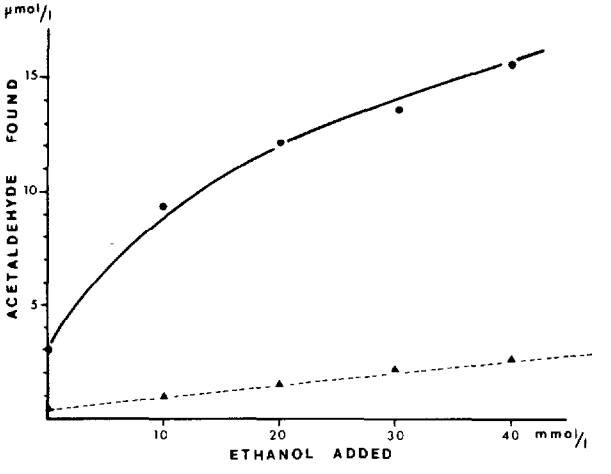

Fig. 5. Acetaldehyde artefact: acetaldehyde production induced by different amounts of ethanol added in vitro for the same blood collected in heparin (●—●) or K_3 EDTA (▲----▲) Vacutainer tubes.

Applications

Acetaldehyde levels were determined in eight fasting controls and fourteen alcoholics and were found to be 0.41 ± 0.13 and 0.91 ± 0.73 μM, respectively (mean ± S.D.) ($p<0.05$). At 30 min after oral administration of ethanol (0.8

g/kg), the ethanol levels were 16.3 ± 2.8 and 17.7 ± 2.5 m*M*, whereas the acetaldehyde levels were 1.67 ± 0.28 and 3.13 ± 1.39 μM ($p<0.05$) for controls and alcoholics, respectively.

The acetaldehyde levels before ethanol administration might correspond to the pathway from threonine, deoxyribose phosphate, alanine and pyruvate, which are regarded as theorically possible sources of acetaldehyde [17]. In alcoholics, the increase in the basic level might be due to residual impregnation with alcohol. After administration of ethanol, the spread values among the alcoholics show the different responses of these patients. Even if the two highest values are excluded, the acetaldehyde levels become 0.68 ± 0.27 μM ($p<0.01$ versus controls) before and 2.30 ± 0.64 μM ($p<0.02$ versus controls) after ethanol administration. Hence these results are not in agreement with those previously reported by Eriksson and Peachey [18], who did not find any difference in the blood acetaldehyde levels of alcoholics and controls after ethanol ingestion, but our results follow those of Palmer and Jenkins [19].

This method is easy to carry out in a routine clinical laboratory and it can measure not only plasma acetaldehyde but also free blood acetaldehyde with minimal interference from ethanol due to the presence of K_3 EDTA in the sample. The procedure is accurate and precise. The lower limit of quantitative detection is improved when microbore HPLC is used. The proposed method principally measures the free acetaldehyde level. However, the interaction and the binding of acetaldehyde to proteins is now well described [1,20] and the level of acetaldehyde bound to proteins should be better known. Using a trapping reagent such as dinitrophenylhydrazine in acidic medium, the determination of the acetaldehyde released from proteins after hydrolysis could be tested, as previously reported for glycosylated haemoglobin [21]. The determination of acetaldehyde bound to haemoglobin is in progress and should complete this study.

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REFERENCES

1 T.M. Donohue, D.J. Tuma and M.F. Sorrell, Lab. Invest., 220 (1983) 239.
2 D.J. Tuma and M.F. Sorrell, Prog. Clin. Biol. Res., 183 (1985) 3.
3 C.J.P. Eriksson, Pharmacol. Biochem. Behav., 18 (1983) 141.
4 C.J.P. Eriksson, Alcoholism: Clin. Exp. Res., 4 (1980) 22.
5 H.W. Sippel, Acta Chem. Scand., 17 (1973) 541.
6 C.J.P. Eriksson, Y. Mizoi and T. Fukunaga, Anal. Biochem., 125 (1982) 259.
7 J.M. Christensen, H. Angelo and J. Knop, Clin. Chim. Acta, 116 (1981) 389.
8 C. Lynch, C.K. Lim, M. Thomas and T.J. Peters, Clin. Chim. Acta, 130 (1983) 117.
9 C. Pezzoli, M. Galli-Kienle, C. Di Padova and G. Stramentinoli, J. Liq. Chromatogr., 7 (1984) 765.
10 A.I. Vogel, Practical Organic Chemistry, Longmans Green, London, 1967, p. 1066.
11 L.A. Curie, Anal. Chem., 40 (1968) 586.

12 R.P.N. Scott, J. Chromatogr. Sci., 23 (1985) 233.
13 G. Chiavari and C. Bergamini, J. Chromatogr., 318 (1985) 427.
14 Y. Hoshika and Y. Takata, J. Chromatogr., 120 (1976) 379.
15 J. Seibl, Helv. Chim. Acta, 50 (1967) 263.
16 H. Buzikiewicz, C. Djerassi and D.H. Williams (Editors), Mass Spectrometry, Holden-Day, San Francisco, CA, 1967, p. 402.
17 C.J.P. Eriksson, Biochem. Pharmacol., 34 (1985) 3979.
18 C.J.P. Eriksson and J.E. Peachey, Pharmacol. Biochem. Behav., 13 (Suppl. 1) (1980) 101.
19 K.R. Palmer and W.J. Jenkins, Gut, 23 (1982) 729.
20 V.J. Stevens, W.J. Fantl, C.B. Newman, R.V. Sims, A. Cerami and C.M. Peterson, J. Clin. Invest., 63 (1981) 361.
21 J.F. Ménez, F. Berthou, A. Meskar, D. Picart, R. Le Bras and L.G. Bardou, J. Chromatogr., 297 (1984) 339.