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DETERMINATION OF PYRROLIDINONE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and selective high-performance liquid chromatographic method for the determination of pyrrolidinone (I) in plasma is described. Compound I was extracted from plasma with methylene chloride using kieselguhr as aqueous phase support. The γ-aminobutyric acid (II) formed after alkaline hydrolysis of I was derivatised with *o*-phthalaldehyde, chromatographed on a reversed-phase column, and quantified by fluorescence detection. The limit of quantification was 2.5 ng/ml (intra-assay R.S.D. 5%) using a 1-ml plasma sample. The inter-assay precision was 3—14% (R.S.D.) over the concentration range 2000—5 ng/ml, and the recovery from plasma was quantitative (99.3 ± 2.9%). The accuracy of this method was established from the good agreement between the values obtained after the analysis of plasma samples by both this method and a gas chromatography—mass spectrometry method.

The high-performance liquid chromatographic procedure was applied to the determination of (a) endogenous I in human plasma samples and (b) I in plasma following intravenous administration of this substance to a dog. In twelve human subjects, endogenous concentrations of I of 8.3 ± 2.3 ng/ml (gas chromatography—mass spectrometry method: 6.1 ± 2.6 ng/ml) were found.

INTRODUCTION

The interest in 2-pyrrolidinone (I, Fig. 1) as far as its possible role in neurotransmission is concerned has increased greatly in recent years. This is due, in part, to its formal relationship to γ-aminobutyric acid (II), and also to its reported occurrence as an endogenous constituent in mammals [1, 2]. In addition, it has been suggested [3] that I is involved in the catabolism of the polyamines; this is in agreement with the observation that I is formed from putrescine in vitro by rat liver preparations [4]. Mori et al. [1] used mass fragmentography for the detection of I in bovine brain, but no details were

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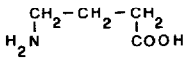
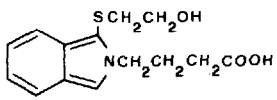
I



II



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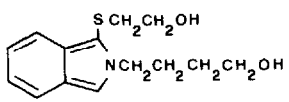

IV

Fig. 1. Structures of pyrrolidine (I), γ-aminobutyric acid (II), the isoindole (III) formed from reaction of OPA reagent with II, and the isoindole (IV) formed from reaction of OPA reagent with 4-amino-1-butanol.

given concerning the precision, accuracy, or sensitivity of this method. Callery et al. [2] described the separation of I from endogenous γ-aminobutyric acid by ion-exchange chromatography, followed by hydrolysis to II and then analysis of the dimethylformamidino derivative by gas chromatography—mass spectrometry (GC—MS). Again, no evidence was presented for the validation of the method.

Recently, in our laboratories, the presence of I in the plasma of humans, rats, and mice was confirmed using a sensitive and specific GC—MS method involving formation of the pentafluorobenzoyl derivative via trimethylsilylation. This method was validated and showed a detection limit for I of 60 pmol/ml of plasma, or 5 ng/ml [5, 6]. In addition to the interest in confirming the presence of endogenous I, a number of studies were envisaged with the objective of determining the pharmacokinetic parameters of I following the oral and intravenous administration of this compound to animals. For the latter, it was considered necessary to have a method that lends itself more to routine determinations than GC—MS. This report describes a high-performance liquid chromatographic (HPLC) procedure, based on the hydrolysis of I to II, followed by derivatisation with *o*-phthalaldehyde (OPA) and fluorescence detection. The accuracy and specificity of this method could be tested by comparison with the results obtained from the GC—MS method.

EXPERIMENTAL

Reagents and materials

OPA, 2-pyrrolidinone, 2-mercaptoethanol, boric acid, 1 *M* sodium hydroxide solution (Titrisol, No. 9956), 1 *M* hydrochloric acid solution (Titrisol, No. 9970), 4-amino-1-butanol (internal standard), disodium hydrogen phosphate,

sodium dihydrogen phosphate, methanol and dichloromethane were all p.a. grade from E. Merck (Darmstadt, F.R.G.) and were used without further purification. Extrelut solid-extraction tubes were also from Merck (Extrelut 1, Cat. No. 15371). Water was distilled twice in all-glass apparatus before use. Borate buffer (pH 9.5) was prepared by mixing 300 ml of 0.4 *M* boric acid with 70 ml of 1 *M* sodium hydroxide.

Preparation of standard solutions

Compound I was dissolved in methanol to give a stock solution (10 mg/ml) from which a range of standards were prepared by diluting this solution with water down to a concentration of 1 ng/ml. Solutions of the internal standard, 4-amino-1-butanol, were prepared in 2 *M* sodium hydroxide at two concentrations, 1.5 μg/ml and 250 ng/ml. The former solution was used for concentrations of I of 50—2000 ng/ml and the latter for 1—100 ng/ml. These internal standard solutions were stable at room temperature for at least one month.

Preparation of OPA reagent

The OPA reagent for fluorescent derivatisation was prepared by dissolving OPA (50 mg) in methanol (100 ml) and adding 0.5 *M* borate buffer (pH 9.5) (20 ml) followed by mercaptoethanol (0.2 ml). This solution was stable for at least one month when stored in a tightly stoppered brown glass vessel at room temperature.

Sample preparation procedure

Before use, the Extrelut columns were washed with 6 ml of methanol, followed by 2 × 5 ml of dichloromethane, and then, finally, dried in a current of air. Frozen plasma samples and aqueous standards were allowed to thaw at room temperature and then homogenised on a vortex mixer. Sample volumes of 1 ml (100 μl for concentrations of I above 2000 ng/ml) of plasma or aqueous standard were applied to an Extrelut column. After 5 min, 3 × 5 ml of distilled dichloromethane were allowed to run through the column, and the extract was then evaporated to dryness using a rotary evaporator (25°C, $0.267 \cdot 10^5$ Pa). Since I is relatively volatile in the dry state, it was important to stop the evaporation as soon as the solvent had disappeared.

Hydrolysis and derivatisation

The procedure for concentrations in the range 50—2000 ng/ml is described, and between parentheses that for 1—100 ng/ml. The residue from the evaporation was dissolved in 1 ml (400 μl) of 2 *M* sodium hydroxide solution containing 1.5 μg/ml (250 ng/ml) internal standard and heated at 100°C for 30 min in tightly stoppered glass vessels. Following neutralisation with 1 ml of 2 *M* hydrochloric acid (200 μl, 4 *M* acid) and dilution with 6 ml of distilled water, 400 μl of this solution were mixed with 600 μl of OPA reagent (vortex). A 10-μl volume of this solution was injected immediately onto the column. For the lower concentration range, the neutralised solution was not diluted; 900 μl of OPA reagent were added, and immediately after mixing and centrifuging for 5—10 sec at 800 *g*, 10 μl were injected.

Chromatographic procedure

The HPLC system consisted of the following components: Model 410 pump, Rheodyne 7125 injector, SFM 23 fluorescence detector (Kontron, Zürich, Switzerland); 250 mm × 4 mm I.D. column, filled with 5-μm Hypersil ODS (Shandon, Runcorn, U.K.); SP 4100 computing integrator and 4100D minifile (Spectra Physics, Basel, Switzerland). The mobile phase was 0.1 *M* phosphate buffer (pH 5)–methanol (50:45), degassed with helium before use. The operating conditions were as follows: excitation and emission wavelengths, 340 and 450 nm, respectively; column at room temperature; flow-rate, 1.0 ml/min. The retention times for the isoindole derivatives of II and the internal standard (III and IV, respectively) were 7.5 and 16.3 min, respectively.

Calibration and calculation

For the calibration, aqueous standards covering the appropriate concentration ranges were used, instead of plasma standards. The reason for this unusual procedure was that, because the level of endogenous I in plasma shows inter-individual variation, the use of one particular plasma or plasma pool for calibration would have lead to inaccurate results for the quantification of I in a plasma sample, or samples, from a different individual. The use of this procedure, not normally acceptable in biological fluid analysis, was validated by means of extraction experiments and the use of a standard addition procedure, described in the discussion section. The best data fit for the calibration was obtained when a weighting factor ($1/y^2$) for both calibration ranges was applied to the linear regression ($r = 0.9989$).

RESULTS AND DISCUSSION

Sample preparation

Compound I is relatively polar, and could not be extracted from aqueous solution efficiently by conventional mixing techniques with chloroform, dichloromethane or ethyl acetate. In order to obtain a quantitative recovery for instance, it was necessary to saturate 1 ml of plasma with sodium chloride and extract three times with 10-ml portions of chloroform using a tumbler extractor; 2.5 h were required to process twenty samples. However, using the Extrelut procedure, the same number of samples was worked up in 30 min. Compound II, the presence of which would give false positives in this method, was not extracted under these conditions.

Hydrolysis and derivatisation

Compound I can be hydrolysed to II under both acidic and alkaline conditions. Alkaline hydrolysis (2 *M* sodium hydroxide) was complete after 30 min (100°C), but with 2 *M* hydrochloric acid, 2.5 h were required for 100% conversion (Fig. 2).

The reaction of OPA with compounds containing a primary amino group in the presence of mercaptoethanol or an alkylthiol at pH 9.5 to give highly fluorescent 1-(2-hydroxyethylthio)- or 1-alkylthio-substituted isoindoles is the basis of many reported methods for the analysis of amines and amino acids [7—12]. Simons and Johnson [13—16] showed that the fluorescent

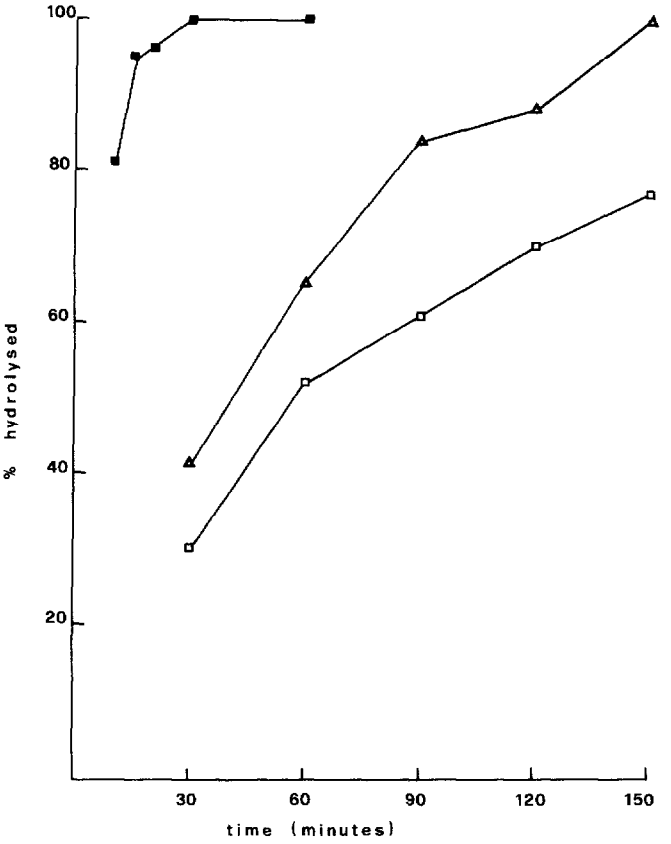

Fig. 2. Hydrolysis of 2-pyrrolidinone to γ-aminobutyric acid at 100°C. Data points: ■ = 2 *M* sodium hydroxide; □ = 1 *M* hydrochloric acid; △ = 2 *M* hydrochloric acid.

product formed from the reaction between OPA and an amine in the presence of mercaptoethanol or ethanethiol was a 1-alkylthio-2-alkyl-substituted isoindole, and this has been confirmed by mass spectrometric studies [17].

Most of the earlier publications for amino acids described post-column derivatisation methods following chromatography on ion-exchange columns. This methodology is somewhat slow and expensive as far as apparatus and materials are concerned. In recent years, there has been a trend towards the use of pre-column derivatisation, using reversed-phase columns for the separation of the fluorescent products; this approach is simpler, more convenient, and can give greater sensitivity.

Stability of the isoindole derivative

A well documented problem associated with this method is the stability of the isoindole, which varies considerably for the different amino acids. Allison et al. [18] investigated the stability of the OPA—2-mercaptoethanol derivatives of nineteen amino acids and found that the isoindole formed from γ-aminobutyric acid was one of the five least stable isoindoles, the others being the derivatives of glycine, taurine, ornithine, and lysine. These five derivatives lost 75—100% of their initial fluorescence response within 40 min at room tem-

perature, in a final reaction solution consisting of a 4:1 mixture of aqueous borate buffer (pH 9.5) and methanol at room temperature. Several authors have found that increasing the proportion of the organic component in the reaction mixture leads to increased stability of the isoindole [11, 12, 19]. Our own investigations clearly support these findings as far as III is concerned (Fig. 3). To a limited extent, the effects of buffer pH and organic component polarity were also studied. It was found that a decrease in the organic solvent polarity enhanced stability, whereas a lowering of the pH from 9.5 to 8.0 had the opposite effect (Fig. 4). The proportion and nature of the organic solvent in the final reaction mixture are, however, limited by mobile phase and buffer compatibility considerations, in so far as the greater the difference between the injection solution and the mobile phase of the ratio alcohol—aqueous solution, the worse the peak shape, leading eventually to double peaking. The optimal conditions were: phosphate buffer—methanol (1:0.9) for the mobile phase, and a 1:1 methanol—aqueous solution injection mixture.

In this injection solution, the degradation rate was 20% per h at room temperature for III and IV. To avoid a degradation of more than 5%, the sample had to be injected within 15 min of derivatisation, which ruled out the use of commercial autoinjectors in their normal mode of operation. Systems have been developed in which reagent and analyte solution are mixed automatically and immediately injected onto the HPLC column. Venema et al. [20] used a peristaltic pump to withdraw an amino acid solution and mix it with the OPA reagent. This mixture was then led into the loop of a pneumatically controlled injection valve for HPLC. In addition, commercial injectors have been modified to perform this task [7, 21].

Recent studies have shown that increasing the size of the thiol used in the OPA reagent improves the stability of the resulting isoindole. Stobaugh et al. [22] and Allison et al. [18] found that the use of 3-mercapto-1-propanol and *tert.*-butylthiol, respectively, gave rise to OPA derivatives that were significantly more stable than those obtained using 2-mercaptoethanol. The OPA—*tert.*-butylthiol derivatives of II, however, exhibited a relative fluorescent intensity

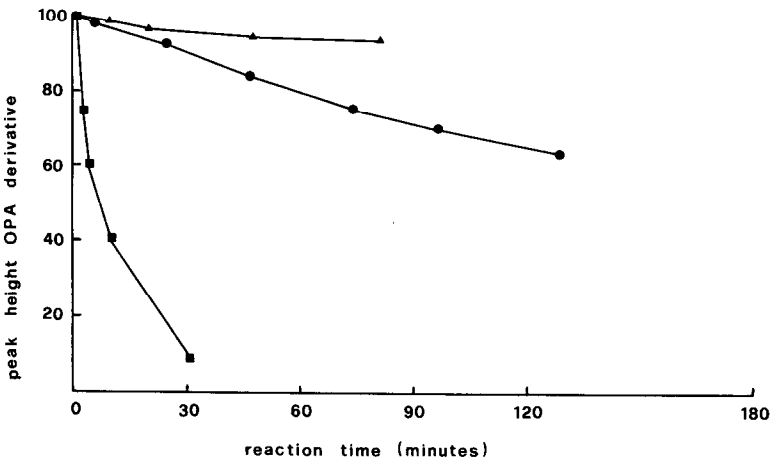

Fig. 3. Stability of III: the influence of the ratio of alcohol to borate buffer (pH 9.5) in the derivatisation mixture at room temperature. Data points: ▲ = 3:1; ● = 5:3; ■ =1:3.

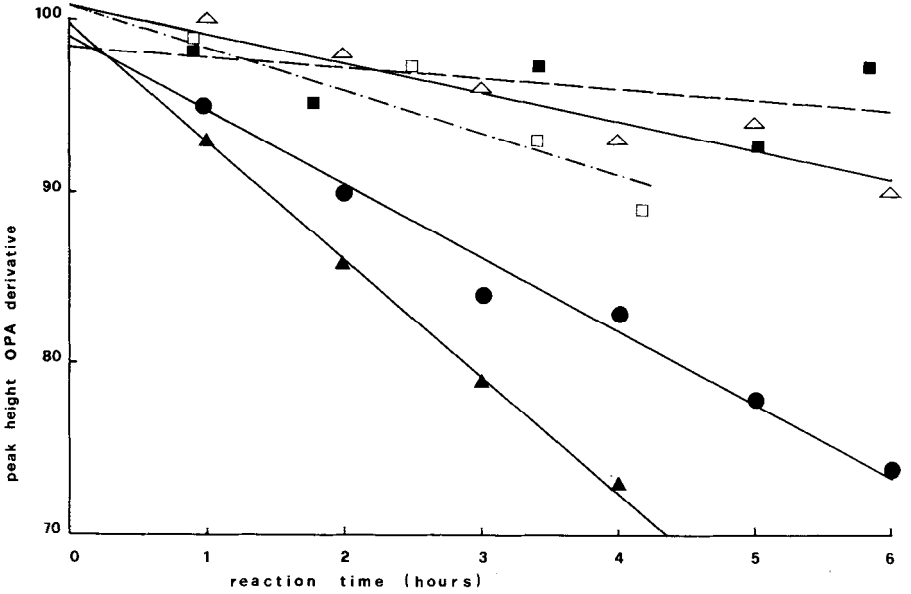

Fig. 4. Stability of III: the influence of the polarity of the organic component and the pH of the buffer in the derivatisation mixture. Data points: △ = methanol–ethanol–borate buffer (pH 9.5) (4:5:1); ■ = methanol—dioxan—borate buffer (pH 9.5) (4:5:1); □ = methanol—dioxan—borate buffer (pH 8.0) (4:5:1); ● = methanol—borate buffer (pH 9.5) (9:1); ▲ = methanol—borate buffer (pH 8.0) (9:1).

that was about six times less than that of the OPA—2-mercaptoethanol derivative [18].

Quantification

Analysis of blank plasma from human subjects indicated the presence of a component that eluted together with III; its concentration, expressed as I, lay between 4 and 14 ng/ml of plasma. This component could not be separated from III on a variety of column types, including non-polar bonded phases (C_{18}, C_8, C_2), polar phases (diol, cyanopropyl, aminopropyl) and strong anion- and cation-exchange columns. The indications were, therefore, that this endogenous component was in fact I, and this was supported by the results of the analysis of the same samples by the more selective GC—MS method already referred to [6]. The mean concentrations of I in plasma samples from twelve human subjects determined by HPLC and GC—MS were 8.3 ± 2.3 and 6.1 ± 2.6 ng/ml, respectively. The HPLC method yielded data that were 1.8 ng/ml higher (95% confidence interval, 0.7—2.8 ng/ml) than those obtained from the GC—MS method, indicating either a methodological bias, or else a greater specificity of the GC—MS procedure. At higher concentrations of I, such as those found in plasma following administration of this substance to dogs, good agreement is obtained between the data from the two methods (Table I).

For the determination of endogenous levels of a substance, the usual approach is the standard addition method, in which various concentrations of the compound are added to the same plasma sample. The linear regression

TABLE I

CONCENTRATIONS OF PYRROLIDINONE IN DOG PLASMA FOLLOWING INTRAVENOUS BOLUS DOSE OF 170 mg IN 0.9% SALINE

Time after injection (min)	Concentration of I (ng/ml)		$\frac{B-A}{A} \cdot 100$
	HPLC (A)	GC–MS (B)	
5	16648	16480	−1%
15	15843	16027	+1%
30	14434	14496	+0.4%
60	12458	12553	+0.8%
90	10504	10210	−3%
120	6845	7539	+10%
150	4027	4419	+10%
180	2179	2295	+5%
240	519	530	+2%
300	122	144	+18%

TABLE II

RECOVERY OF PYRROLIDINONE FROM PLASMA AND WATER

Concentration (ng/ml)	Percentage recovery (mean ± S.D., n=3)	
	Water	Plasma
20	100.3 ± 1.2	99.0 ± 1.7
100	97.0 ± 1.0	102.7 ± 0.6
2000	94.7 ± 0.6	96.3 ± 0.6

(analytical response versus concentration) obtained from the analysis of these samples would give an intercept corresponding to the endogenous concentration. A disadvantage of this approach is that it requires relatively large volumes of blood. For instance, on the basis of five concentrations, 1 ml of plasma for each determination and duplicate analyses, ca. 25 ml of blood would be required. Although this is no problem for human volunteers, taking such a volume from individual laboratory animals would be either undesirable or impracticable.

It was therefore decided to investigate the feasibility of using aqueous standards for calibration, although this would not normally be acceptable for biological fluid analysis. To test the validity of this approach, it was necessary, above all, to demonstrate that the recovery of I from water was the same as that from plasma, and that the background interference from water was negligible (see below under *precision, sensitivity and linearity*). The recovery was studied by spiking water and control plasma with I at three different concentrations, extracting and analysing these samples by the method described, and comparing the resulting peak heights with the "100%" values. The latter were obtained by hydrolysing aqueous solutions of I followed by derivatisation. The data in Table II indicate that the recoveries from both water and plasma were quantitative in the concentration range 20–2000 ng/ml. For the 20 and 100 ng/ml samples, the peak heights of endogenous I in plasma were taken into account in the calculations. Further evidence for the validity of this standardisation method came from the repeated analysis of quality control

TABLE III

ANALYSIS OF PLASMA QUALITY CONTROL SAMPLES USING AQUEOUS STANDARDS OVER A PERIOD OF ONE MONTH: INTER-ASSAY PRECISION AND ACCURACY

Concentration added (ng/ml)	Mean concentration found (ng/ml)*	*n*	R.S.D.	$\frac{\text{found} - \text{added}}{\text{added}}$ (%)
5	4.9	6	14	−2
20	19.2	7	6	−4
100	94.1	5	7	−6
500	477	5	3	−5
1000	968	5	9	−3
2000	2014	5	3	+0.7

*After allowing for endogenous I, determined at the same time.

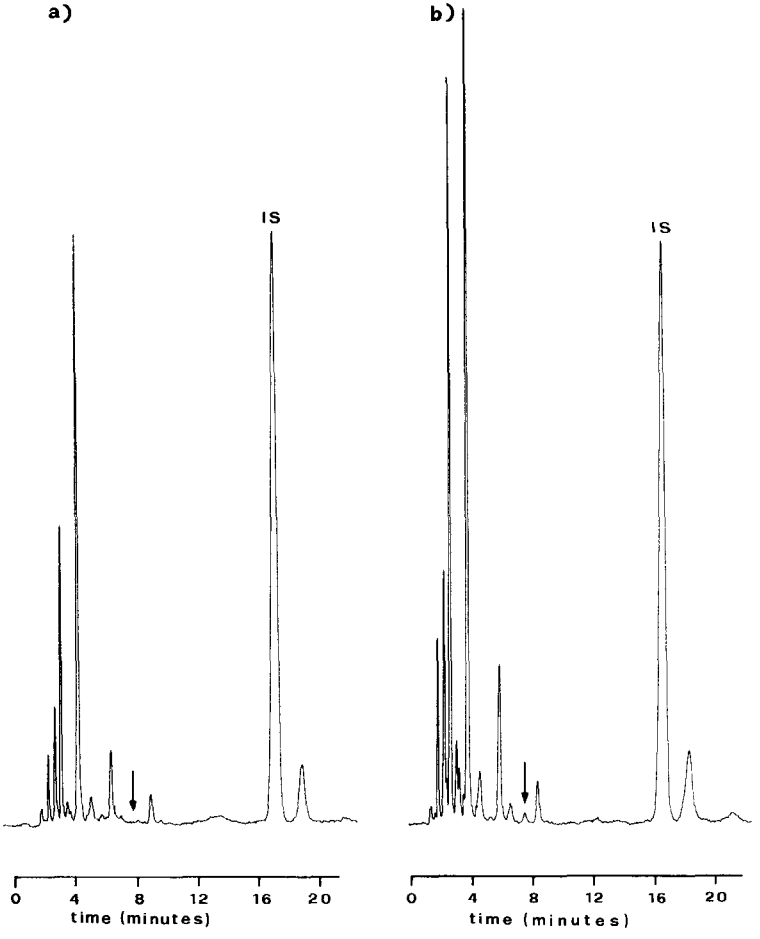

Fig. 5. (a) Reagent blank chromatogram: dichloromethane evaporated, the residue subjected to the hydrolysis conditions and then the derivatisation procedure. (b) Extrelut blank chromatogram: 1 ml of water applied to Extrelut column, eluted with 3 × 5 ml dichloromethane and then as (a). The arrows indicate the retention time of III. In (b), this peak is equivalent to 0.3 ng/ml of I. IS = internal standard.

plasma samples using the aqueous standards over a one-month period (Table III). Finally, good agreement was obtained for the analysis of endogenous I in a plasma sample between the results using aqueous standardisation and a standard addition method (see below).

Standard addition. Blank human plasma was spiked with I in concentrations of 1, 3, 10, 50, and 100 ng/ml; samples were analysed on three separate occasions and the following linear regressions ($1/y^2$ weighting factor) were obtained (x = response, y = concentration): $y = 54.9x - 9.1$; $y = 58.1x - 9.5$; $y = 57.8x - 9.7$. The mean intercept, corresponding to endogenous I, was 9.4 ng/ml (R.S.D. 3.3%).

Aqueous calibration. The same blank plasma (no I added) was analysed, using aqueous calibration standards, on eight separate occasions; the following

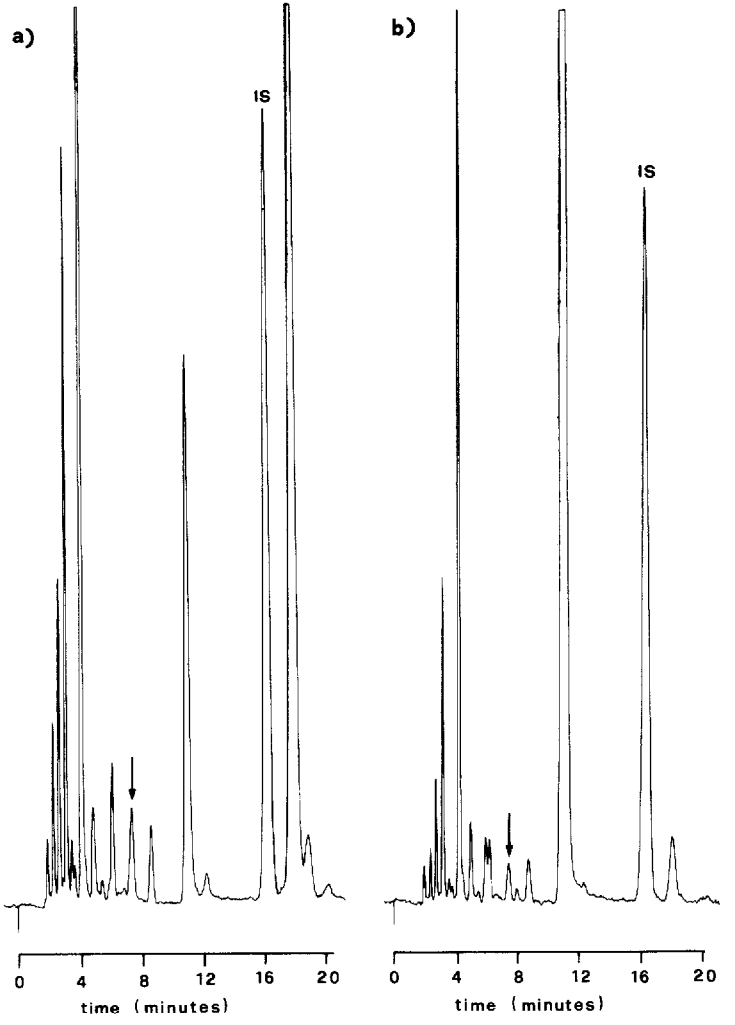

Fig. 6. Chromatograms of (a) blank human plasma and (b) blank dog plasma. The arrows indicate the peak corresponding to III. This is equivalent to 5.0 and 2.5 ng/ml of I, in (a) and (b), respectively.

concentrations were calculated (ng/ml): 11.0, 10.6, 9.7, 9.1, 9.3, 9.9, 9.3, and 8.7; the mean value was 9.7 ng/ml (R.S.D. 8.0%).

Precision, sensitivity and linearity

The quality control samples referred to above were used to assess the inter-assay precision during one month. For the concentration range 5—2000 ng/ml, the R.S.D. was 3—14%. The washing procedure for the Extrelut columns reduced the background interference from 10 to 0.3 ng/ml (Fig. 5b), allowing a detection limit of ca. 1 ng/ml. Omitting the Extrelut step, dichloromethane was evaporated and the residue subjected first to the hydrolysis, and then to the derivatisation procedure; this indicated that the interference originated from the Extrelut itself (Fig. 5a). The lowest concentrations encountered were 2.5 ng/ml (intra-assay R.S.D. 5%, n=6, Fig. 6b) from dog plasma, and 5.0 ng/ml (inter-assay R.S.D. 12%, n=5, Fig. 6a) from human plasma. The response was linear for up to 2 ng equivalents of I injected, and the best data fit (correlation coefficient 0.9989) was obtained using a weighted linear regression ($1/y^2$). The sensitivity is comparable with that of the HPLC method for II recently described by Yamamoto et al. [23], which involves derivatisation of II with 2,4,6-trinitrobenzenesulphonic acid followed by dual electrochemical detection.

Stability

The stability of I in plasma at room temperature for 24 h, and at −20°C for three months, was investigated at three different concentrations. The analytical responses of the stored samples were compared with those of samples prepared on the day of analysis; the 90% confidence interval for the change of concentration after storage was calculated according to the procedure described in detail elsewhere [24]. In addition, the stability of a 50 ng/ml sample in human whole blood after 4 h at room temperature was studied. Under all these conditions, no serious instability was observed (Table IV).

TABLE IV

STABILITY OF PYRROLIDINONE IN PLASMA AND WHOLE BLOOD

Sample concentration (ng/ml)	Storage conditions	Change of concentration* after storage (%)	90% Confidence interval for change of concentration (%)
Plasma			
200	24 h at 25°C	−2.1	−0.7 to −3.4
	3 months at −20°C	+0.2	+2.6 to −2.2
1000	24 h at 25°C	+0.3	+2.3 to −1.7
	3 months at −20°C	−0.6	+1.5 to −2.7
4000	24 h at 25°C	−1.3	+1.6 to −4.0
	3 months at −20°C	+0.2	+3.0 to −2.7
Whole blood			
100	4 h at 25°C	+0.5	+2.7 to −1.6

*Five replicate determinations.

Application

Plasma samples from a dog that had received an 11.7 mg/kg dose of I were analysed by both the HPLC and the GC—MS methods (Table I), as were control plasma samples from twelve human subjects (see *Quantification*). The good overall agreement between the data over a wide concentration range is an indication of the accuracy of the two methods.

One of our sources of control plasma was blood bank material from a local hospital, collected into plastic bags. The plasma from this source contained an interference, amounting to ca. 5 ng/ml equivalents of I. The source of this background was traced to the plastic bags. All vessels used in this determination should be routinely checked for interference; it was found that blood or plasma collected and stored in glass containers was free of interference coming from the vessels themselves.

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REFERENCES

1 A. Mori, Y. Katayama, M. Matsumoto and H. Takeuchi, IRCS Med. Sci., 3 (1975) 590.
2 P.S. Callery, L.A. Geelhaar and M. Stogniew, Biochem. Pharmacol., 27 (1978) 2061.
3 N. Seiler, Physiol. Chem. Phys., 12 (1980) 411.
4 D.W. Lundgren and J. Hankins, J. Biol. Chem., 253 (1978) 7130.
5 E.F. Bandle, G. Wendt, U.B. Ranalder and K.-H. Trautmann, Life Sci., 5 (1984) 2205.
6 G. Wendt, U.B. Ranalder and K.-H. Trautmann, in preparation.
7 J.C. Hodgin, P.Y. Howard, D.M. Ball, C. Cloete and L. De Jager, J. Chromatogr. Sci., 21 (1983) 503.
8 H. Umagat, P. Kucera and L.F. Wen, J. Chromatogr., 239 (1982) 463.
9 F.H. Walters and K.B. Griffin, Anal. Lett., 16(A6) (1983) 485.
10 G.R. Barbarash and R.H. Quarles, Anal. Biochem., 119 (1982) 177.
11 S.S. Simons and D.F. Johnson, Anal. Biochem., 90 (1978) 705.
12 T. Skaaden and T. Greibrokk, J. Chromatogr., 247 (1982) 111.
13 S.S. Simons, Jr. and D.F. Johnson, J. Amer. Chem. Soc., 98 (1976) 7098.
14 S.S. Simons, Jr. and D.F. Johnson, J. Chem. Soc. Chem. Commun., (1977) 374.
15 S.S. Simons, Jr. and D.F. Johnson, Anal. Biochem., 82 (1977) 250.
16 S.S. Simons, Jr. and D.F. Johnson, J. Org. Chem., 43 (1978) 2886.
17 R.C. Simpson, J.E. Spriggle and H. Veening, J. Chromatogr., 261 (1983) 407.
18 L.A. Allison, G.S. Mayer and R.E. Shoup, Anal. Chem., 56 (1984) 1089.
19 R.F. Chen, C. Scott and E. Trepman, Biochim. Biophys. Acta, 576 (1979) 440.
20 K. Venema, W. Leever, J.D. Bakker, J.O. Haayer and J. Korf, J. Chromatogr., 260 (1983) 371.
21 M.O. Fleury and D.V. Ashley, Anal. Biochem., 133 (1983) 330.
22 J.F. Stobaugh, A.J. Repta, L.A. Sternson and K.W. Garren, Anal. Biochem., 135 (1983) 495.
23 T. Yamamoto, C. Nanjoh and I. Kuruma, Neurochem. Int., 7(1) (1985) 77.
24 U. Timm, M. Wall and D. Dell, J. Pharm. Sci., in press.