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DETERMINATION OF CYANAMIDE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, selective and rapid high-performance liquid chromatographic procedure was developed for the determination of cyanamide in plasma. The procedure involved extraction with ethyl acetate, derivatization with 5-(dimethylamino)naphthalene-1-sulphonyl chloride and posterior analysis by high-performance liquid chromatography on a μBondapak C_{18} column with fluorimetric detection. Linearity ranged from 5 to 500 ng/ml and the lower limit of sensitivity of the assay was 4 ng/ml of cyanamide in plasma. The precision of the method was 3.0–8.9%, expressed as relative standard deviation over the linear range. This method has been used to elucidate the time course of the cyanamide concentration in the plasma of humans, following oral administration of cyanamide at therapeutic doses.

INTRODUCTION

Calcium cyanamide in the citrated form (Temposil®, Abstem®, Dipsan®) or as an aqueous cyanamide solution (Colme®) is commonly referred to as an antialcohol, an alcohol deterrent or an alcohol-sensitizing drug, due to the detrimental effects produced during its interaction with ethanol [1]. During the cyanamide–ethanol reaction, the acetaldehyde concentration in the blood is increased [2], which results in a number of undesirable effects such as tachycardia, hypotension, flushing and disnea, thereby deterring further drinking.

Cyanamide inhibits aldehyde dehydrogenase (EC 1.2.1.3) [3], one of the enzymes involved in ethanol metabolism, and this inhibition induces an increase in acetaldehyde levels in the blood.

There are very few analytical methods for the measurement of cyanamide in biological fluids. For this purpose, these methods involve spectrophoto-

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metric [4, 5] and gas chromatographic techniques [6]. However, the former technique [4, 5] is not sensitive enough and the gas chromatographic technique [6] was not reproducible in our hands.

In this paper, we describe a very sensitive and selective method for the measurement of cyanamide in plasma. The procedure involves the extraction with ethyl acetate from plasma, cyanamide derivatization with 5-(dimethylamino)naphthalene-1-sulphonyl chloride (dansyl chloride) and posterior chromatography on a reversed-phase C_{18} column with fluorimetric detection. The sensitivity of this method allowed us to follow the extremely low cyanamide concentrations in the plasma of humans after a single therapeutic dose.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile (HPLC grade) was purchased from Farmitalia Carlo Erba (Milan, Italy). Acetone was analytical reagent grade and obtained from Koch-Light (Haverhill, U.K.). The following reagents and materials were from Merck (Darmstadt, F.R.G.): acetic acid, methanol, sodium bicarbonate, sodium carbonate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate (reagent grade), dansyl chloride (for fluorescence of amino acids), ethyl acetate ("LiChrosolv", which was used without further purification) and Kieselgel 60 thin-layer chromatographic plates. Cyanamide was obtained from Fluka (Buchs, Switzerland). Solvents for high-performance liquid chromatography (HPLC) were filtered through a Millipore 0.5-μm filter and thoroughly degassed in an ultrasonic bath before use. The water used was bidistilled and purified through a Milli-Q system (18 MΩ cm resistivity).

Apparatus

Chromatographic separations were carried out on a Waters gradient HPLC system (Waters Assoc., Milford, MA, U.S.A.) equipped with two M-510 solvent delivery systems, an M-721 solvent programmer, a WISP 710B automatic injector and an M-420 AC fluorescence detector with a 10-μl flow-through cell. A 360-nm (bandpass) excitation wavelength was selected in conjunction with a 495-nm (longpass) emission filter and an F4T5/BL lamp as a light source. Peak areas were integrated with an M-730 data module (Waters Assoc.).

TABLE I

SOLVENT PROGRAMME DELIVERY SYSTEM

Time (min)	Flow-rate (ml/min)	Solvent A (%)	Solvent B (%)	Curve
0	4	70	30	—
7	4	40	60	6
10	4	0	100	1
12	4	70	30	1

Chromatography

The column was a μBondapak C_{18} Radial-Pak cartridge (10 × 0.8 cm I.D., particle diameter 10 μm) obtained from Waters Assoc. and used in conjunction with a Z-module radial compression separation system. A Guard-Pak precolumn insert packed with μBondapak C_{18} was used to protect the column. The mobile phase consisted of two solvents; solvent A was 10 m*M* potassium phosphate (pH 7.0) and solvent B was acetonitrile—10 m*M* potassium phosphate (pH 7.0) (55:45). The solvent programme delivery system that was used is given in Table I. The guard and analytical column were at room temperature.

Standard and sample preparations

A stock solution of cyanamide (1 mg/ml) was prepared in water. Aqueous standards in the concentration range 25—2500 ng/ml were prepared by dilution of the stock solution with water. To 0.5-ml mixed citrated plasma samples from untreated rats placed in 10-ml silanized glass centrifuge tubes fitted with PTFE-lined screw caps were added 0.1-ml volumes of different cyanamide aqueous standards, to obtain a calibration curve in the concentration range 5—500 ng cyanamide per ml plasma (0.1 ml of water to the plasma blank and the treated plasma samples). Then 2.0 ml of ethyl acetate were added and the tubes were shaken for 30 s in a vortex mixer and centrifuged at 2000 *g* for 5 min. Aliquots (1.5 ml) of the organic phases were transferred to 4-ml silanized conical glass reacti-vials. A re-extraction was made by adding 2.0 ml of ethyl acetate and then 2.0 ml of the organic phases were taken out and added to the previous organic phases. The combined organic extracts were evaporated to dryness with a stream of nitrogen at 40°C and the residue was redissolved in 100 μl of 0.2 *M* sodium carbonate- sodium bicarbonate (pH 9.0) buffer solution. Derivatization was carried out by adding 100 μl of dansyl chloride solution (1 mg/ml in acetone) to the tubes, which were capped with a PTFE-lined screw cap and mixed with a vortex mixer. The tubes were kept at 40°C for 1 h to complete dansylation. After cooling to room temperature, 50 μl were injected onto the column. The fluorescence obtained was stable for at least 2 h. Cyanamide plasma samples can be stored frozen at - 80°C for at least two weeks without any significant loss of the compound.

Quantitation

Samples in the concentration range 5—500 ng/ml were estimated by direct interpolation from the calibration curve; samples over 500 ng/ml were diluted in acetone—0.2 *M* bicarbonate—carbonate (pH 9.0) buffer solution (1:1) to reach a concentration level that can be interpolated from the curve.

Human plasma sampling

The method was applied to follow the time course of cyanamide concentration in the plasma of one volunteer. A 35-year-old man weighing 60 kg received, under fasting conditions, 1 mg/kg cyanamide as an oral aqueous solution. Before administration, a 20-ml sample of blood was drawn to prepare the blank plasma and the calibration curve. Blood samples were taken at 5, 10, 20, 30. 40, 50, 60, 90, 120, 150, 180, 240, 360 and 480 min after oral administration of cyanamide and were centrifuged at 1000 *g*. Plasma was removed and

maintained at −80°C until analysis was performed the next day. Analysis of samples were carried out according to the previously described method.

RESULTS

Linearity

A straight line was obtained by plotting the values of cyanamide in rat plasma (ng/ml) against the peak areas. The results from five standard curves obtained in three consecutive days are listed in Table II (correlation coefficients ranged from 0.9995 to 1.0000). The relationship was linear in the concentration range 5–500 ng/ml of cyanamide in plasma.

TABLE II

MEANS, STANDARD DEVIATIONS (S.D.) AND RELATIVE STANDARD DEVIATIONS (R.S.D.) OF PEAK AREA OF CYANAMIDE DANSYLATED DERIVATIVE IN RAT PLASMA

Concentration (ng/ml)	Mean peak area* (n = 5)	S.D.	R.S.D. (%)
5	2.560	0.299	11.7
25	7.033	0.822	11.7
50	13.415	1.302	9.7
100	27.262	2.180	8.0
500	137.341	9.756	7.1

*Arbitrary units.

Recovery

A recovery study was made by adding known amounts of cyanamide to the rat plasma, to obtain cyanamide concentrations of 25, 100 and 500 ng/ml. The values obtained were compared with the same quantities added to physiological media of 100 m*M* sodium phosphate buffer (pH 7.4). Recoveries were 119, 106 and 102%, respectively (n = 7).

Precision

Bulk control standards were prepared by adding known amounts of cyanamide to blank rat plasma to obtain concentrations of 25, 100 and 500

TABLE III

MEANS, STANDARD DEVIATIONS (S.D.) AND RELATIVE STANDARD DEVIATIONS (R.S.D.) DERIVED FROM A WITHIN-RUN PRECISION STUDY

Concentration (ng/ml)	Mean peak area* (n = 5)	S.D.	R.S.D. (%)
25	7.087	0.628	8.9
100	30.125	1.638	5.4
500	143.449	4.233	3.0

*Arbitrary units.

TABLE IV

MEANS, STANDARD DEVIATIONS (S.D.) AND RELATIVE STANDARD DEVIATIONS (R.S.D.) OF BACK-CALCULATED VALUES OF STANDARD CURVES OF CYANAMIDE IN RAT PLASMA

Actual concentration (ng/ml)	Mean concentration found (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Difference from theory (%)
5	6.7	2.1	31.6	34.0
25	24.6	3.3	13.6	1.6
50	49.6	3.2	6.5	0.8
100	98.7	5.7	5.8	1.3
500	500.5	1.1	0.2	0.1

ng/ml. The within-run precision of the assay was determined by analysing samples of each of these controls in quintuplicate and comparing the peak areas obtained. Relative standard deviations (R.S.D., Table III) ranged from 8.9% at the lowest concentration to 3.0% at the highest concentration. Variability of back-calculated concentration values (i.e. concentration values estimated from the corresponding standard curve equations) for each theoretical concentration level are summarized in Table IV. Mean concentration values, standard deviations, relative standard deviations and relative errors from five standard curves obtained at three consecutive days are shown. Relative standard deviations ranged from 31.6% at 5 ng/ml to 0.2% at 500 ng/ml. The percentage difference between the calculated and the theoretical values ranged from 34% to 0.1%.

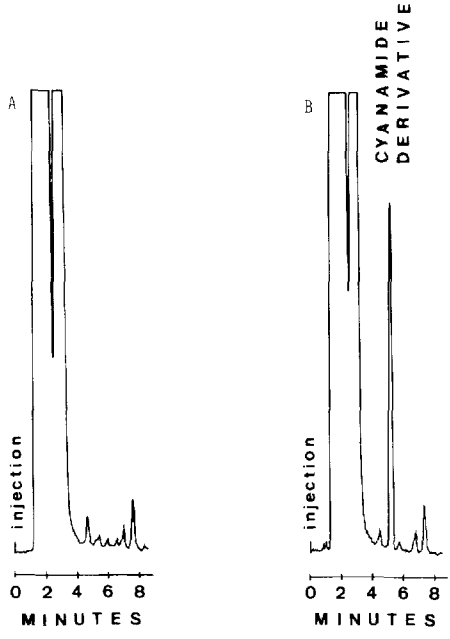

Fig. 1. Chromatograms of dansyl cyanamide derivative in human plasma. (A) Control plasma; (B) plasma sample from a subject 1 h after being given a single oral dose of cyanamide (1 mg/kg). Cyanamide concentration was found to be 125 ng/ml.

Selectivity and sensitivity

Very few (and irrelevant) interfering peaks were visible in blanks of plasma at the retention time of the cyanamide dansylated derivative (retention time 5.2 min).

The minimum detectable cyanamide concentration in plasma was ca. 4 ng/ml. This represents the concentration of compound that produces a peak area equal to the mean blank value plus three standard deviations.

Time course of cyanamide concentration in plasma of humans

The method provided a selective and sensitive procedure for the measurement of cyanamide levels in the plasma of humans. Fig. 1 shows chromatograms of control human plasma (blank sample, Fig. 1A) and cyanamide dansylated derivative in plasma taken 1 h after a single oral dose of 1 mg/kg cyanamide (Fig. 1B). The procedure described here allowed us to follow the cyanamide plasma levels for at least 8 h after oral administration of a therapeutic dose of this drug (1 mg/kg). Fig. 2 shows the time course of cyanamide levels in the plasma of a human volunteer. The peak plasma level (C_{max}) was found to be 1024 ng/ml, which was reached 10 min (T_{max}) after cyanamide administration. The half-life ($t_{½}$) of the compound was found to be ca. 2 h.

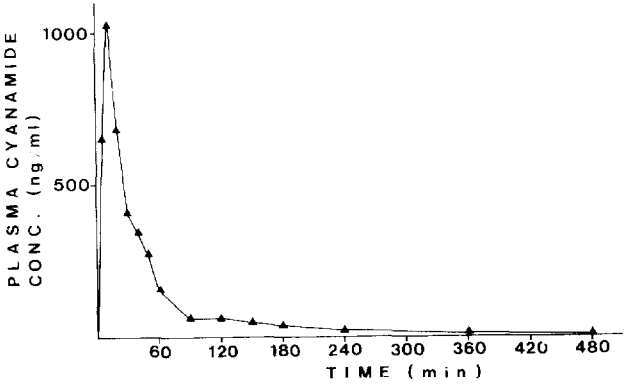

Fig. 2. Time course of human plasma levels of cyanamide after administration of a single oral dose of 1 mg/kg aqueous cyanamide solution.

DISCUSSION

The reported method involves the quantitative determination of cyanamide in plasma as the sodium salt of its dansyl derivative using HPLC with fluorimetric detection. A molar ratio of dansyl chloride to cyanamide of ca. 30:1 has been found to provide maximal derivatization of the most concentrated cyanamide samples.

The dansyl cyanamide derivative obtained, dissolved in diethyl ether saturated with hydrochloric acid to obtain the cationic form, was studied by mass spectrometry to elucidate its structure; the results agreed well with the postulated theoretical structure. The *m/e* fragment ion values found were 276, 250, 105, 64 and 43 (Fig. 3).

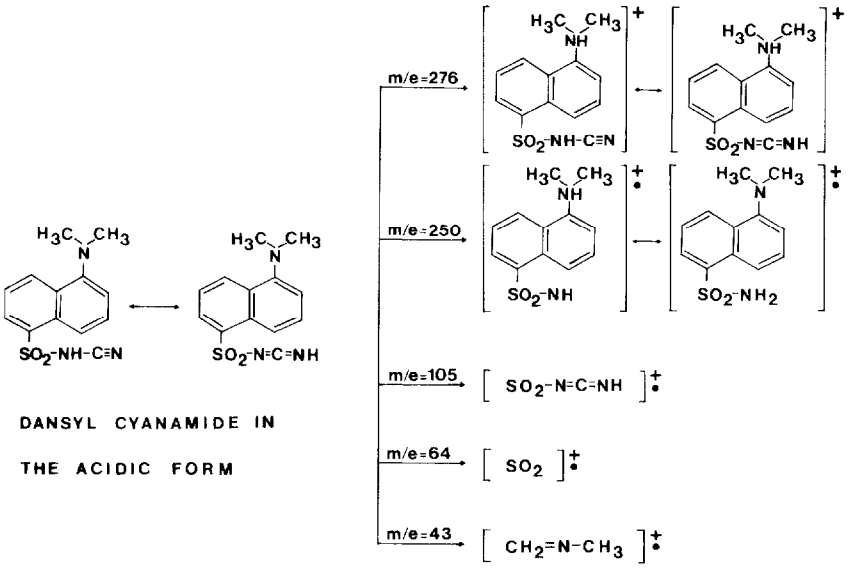

Fig. 3. Chemical structure of dansyl cyanamide derivative and fragment ions determined by mass spectrometry.

The sodium salt of the dansyl cyanamide derivative showed a single spot (R_F = 0.20) when analysed by thin-layer chromatography (TLC) using a Kieselgel 60 plate and ethyl acetate—methanol—acetic acid (100:5:5) as mobile phase. The compound also showed a single chromatographic peak in HPLC analysis at the characteristic retention time (5.2 min), under the conditions described previously.

This method does not include an internal standard. The use of a reliable internal standard would probably improve the reproducibility of the method. For this purpose several compounds were tested, including glycinonitrile, ethylamine, propylamine, isopropylamine, β-aminopropionitrile, thiourea, thioacetamide and α-phenylglycine; however, none of them possessed the appropriate requirements.

In an attempt to improve the cyanamide extraction from plasma the aqueous phase was saturated with sodium chloride at pH 10 as described by Loomis and Brien [6]; however, using these conditions, interfering chromatographic signals were enhanced and thus this attempt was not considered anymore.

The high recovery values observed could be due to differences between the compositions of the aqueous media (plasma and physiological phosphate buffer) used in the recovery study.

The chromatographic resolution of this method is clearly superior to that obtained with the gas chromatographic method [6] (cf. Fig. 1 of the present study with Fig. 1 in ref. 6). This method has been routinely used in our laboratories for the analysis of cyanamide in plasma and is sufficiently accurate and sensitive to be used in pharmacokinetic studies (unpublished results).

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