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Liquid chromatographic method for the determination of calcium cyanamide using pre-column derivatization

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ABSTRACT

A specific and stability-indicating high-performance liquid chromatographic (HPLC) method has been developed for the analysis of calcium cyanamide in bulk material and dosage form. Calcium cyanamide in samples was converted into dansyl cyanamide. A μBondapak C_{18} column was employed for HPLC with 0.01 *M* sodium phosphate (pH 6.3)–acetonitrile (75:25, v/v) as the mobile phase. The proposed HPLC method was validated for linearity, specificity, accuracy and reproducibility.

INTRODUCTION

Calcium cyanamide in the citrated form (Temposil, Dipsan) was introduced into medicine in 1956 as a pharmacological adjunct in the treatment of chronic alcoholism [1]. The drug blocks ethanol metabolism by inhibition of aldehyde dehydrogenase which increases both hepatic and blood acetaldehyde levels after ingestion of ethanol [2,3]. Increased acetaldehyde levels result in a number of undesirable effects such as tachycardia, hypotension, flushing and disnea [4,5].

A limited number of analytical procedures are available in the literature for the quantitation of calcium cyanamide. These methods involve spectrophotometric, paper chromatographic and gas–liquid chromatographic assays [6–9]. A liquid chromatographic procedure for the determination of cyanamide in biological fluids has recently been published in the literature [10]. The method currently used in industry requires a titrimetric assay consisting of an ammonia–silver nitrate reagent precipitation followed by a titration of the silver in the reagent.

Calcium cyanamide is one of many compounds that cannot be readily analyzed directly by high-performance liquid chromatography (HPLC) using spectrophotometric detection. This problem can be overcome by derivatization to introduce a chromophore or fluorophore. The present paper describes the application of dansyl chloride as a pre-column derivatization agent for cyanamide. The assay is a more reliable analytical method for calcium cyanamide in bulk material and dosage form. The method also offers the advantage of monitoring the stability of the citrated formulation with reasonable simplicity, selectivity and specificity.

EXPERIMENTAL

Chemicals and reagents

Calcium cyanamide and Temposil were obtained from American Cyanamid Company (Baie d'Urfe, Canada). Sodium acetate, glacial acetic acid, sodium carbonate, dibasic potassium phosphate (all ACS reagent grade) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Dansyl chloride was purchased from Aldrich (Milwaukee, WI, USA). All organic solvents were of HPLC grade and were obtained from Burdick & Jackson Labs. (Muskegon, MI, USA). The water used was purified through a Milli-Ro-Milli-Q system (Millipore, Bedford, MA, USA). All chemicals and solvents were used as received without any further purification.

Apparatus and chromatographic conditions

Chromatographic separations were carried out on an HPLC system consisting of an SSI 222B pump (State Scientific Instruments, State College, PA, USA), a Waters WISP 712 automatic injector (Waters Assoc., Milford, MA, USA), and a Kratos 783 (Applied Biosystems, Foster City, CA, USA) variable-wavelength UV detector set at 254 nm. The detector's 0–10 mV analog signal was recorded with a Model 1200 chart recorder (Linear Instruments, Reno, NV, USA) and the 0–1 V signal was digitized and recorded with a Nelson Analytical Data system at a sampling rate of one point per second.

All chromatographic procedures were performed at ambient temperature. Injections were made onto a Waters μBondapak C_{18} analytical column (150 mm $\times$ 39 mm I.D., 10 μm particle size). The column was equilibrated with mobile phase at a flow-rate of 1 ml/min. The relative standard deviation (R.S.D.) of six replicate injections of a standard was not more than 2%, as defined in the USP XXII under "System suitability for HPLC" [11]. The detection wavelength and the sensitivity were set at 254 nm and at 0.05 a.u.f.s., respectively.

Standard preparation

A 25-mg weight of calcium cyanamide of known purity was accurately weighed and quantitatively transferred to a 100-ml volumetric flask. To it were added 20 ml of buffer solution for extraction. The buffer solution was made by dissolving 12.0 g of anhydrous sodium acetate in a mixture of 980 ml of water and 20 ml of glacial acetic acid. The calcium cyanamide mixture was sonicated for 10 min for complete dissolution and then diluted to volume with 0.2 *M* sodium carbonate. The flask was allowed to stand at room temperature for 15 min. The clear part of the solution was withdrawn for derivatization.

Sample preparation

Bulk material. A 25-mg calcium cyanamide sample was accurately weighed and transferred to a 100-ml volumetric flask. The sample was treated subsequently in the same manner as standard.

Tablets. Twenty tablets were accurately weighed and the average tablet weight was determined. The tablets were ground to a fine powder and a portion of the tablet powder equivalent to one-half tablet weight was weighed and transferred quantitatively to a 100-ml volumetric flask. The powder was treated in the same manner as the standard.

Derivatization reaction

With a pipettor, 200 μl of the clear part of the solution described in *Standard preparation* and *Sample preparation* were accurately withdrawn and transferred to a screw-cap vial (7.4 ml capacity, Supelco, Bellefonte, PA, USA). Using a pipettor, 200 μl of the derivatizing agent was added and the solution was mixed well. The derivatizing agent was prepared by dissolving 100 mg of dansyl chloride in 10 ml of acetone. The vial was heated in a heating block at 40°C for 30 min and cooled the vial to room temperature. A 4-ml volume of mobile phase was accurately added to the vial and mixed. An aliquot of the solution was transferred to an HPLC vial and 10 μl were injected onto the column.

RESULTS AND DISCUSSION

A selective, sensitive, and specific reversed-phase HPLC assay was developed for the determination of calcium cyanamide in bulk raw material and in its citrated formulation. Calcium cyanamide is dissolved in a sodium acetate buffer (pH $\approx$ 4.3) solution and subsequently hydrolyzed to cyanamide. The cyanamide in a basic solution is reacted with dansyl chloride to form dansyl cyanamide which can be analyzed quantitatively by reversed-phase HPLC (Fig. 1).

A pure dansyl cyanamide was synthesized and evaluated using spectroscopic techniques. The synthesized dansyl cyanamide was characterized by the following spectroscopic data: UV λ_{max} 326 nm (ε 4800), 246 nm (ε 15 000); Fourier transform IR ν 3060 (aromatic H), 2960 (CH_3), 2180 (sharp s, $-C\equiv N$), 1600, 1540 (aromatic); ^1H NMR (in [$^2H_6$]dimethyl sulfoxide, d_6-DMSO) δ 3.15 (s, 6H, $N-(CH_3)_2$, 7.7 (m, 3H, aromatic), 8.15 (d, J = 7.2, 1H, aromatic), 8.39 (d, J = 8.5, 1H, aromatic), 8.72 (d, J = 7.9, 1H, aromatic); chemical ionization mass spectrometry (ammonia) m/z 293 ($M + NH_4$), 276 ($M + H$), 251 ($M - CH_3 + H$). The elemental analysis of dansyl cyanamide confirmed its composition: C 56.35%, H 4.68%, N 15.12%, S 11.64% (theoretical: C 56.71%, H 4.76%, N 15.24%, S 11.65%). The material is also fluorescent with excitation wavelength at 324 nm and emission wavelength at 520 nm. The material was found to be pure (HPLC purity of 97.2% by area percent).

The chemical shift of the N-methyl group of dansyl cyanamide in d_6-DMSO was at relatively low-field (3.18 ppm) as compared to the N-methyl absorption (2.82 ppm) of the model compound, dansylamide. When one drop of NaO^2H was added to the

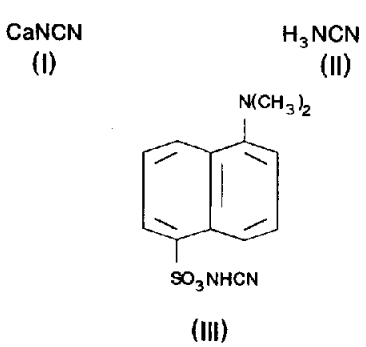

Fig. 1. Structures for calcium cyanamide (I), cyanamide (II) and dansyl cyanamide (III).

NMR tube, the N-methyl absorption of dansyl cyanamide shifted from 3.18 to 2.82 ppm. On the other hand, when one drop of trifluoroacetic acid was added to the NMR tube of dansylamide, the N-methyl absorption shifted from 2.82 to 3.08 ppm. The NMR data thus indicate the formation of a zwitterion in the solution of dansyl cyanamide.

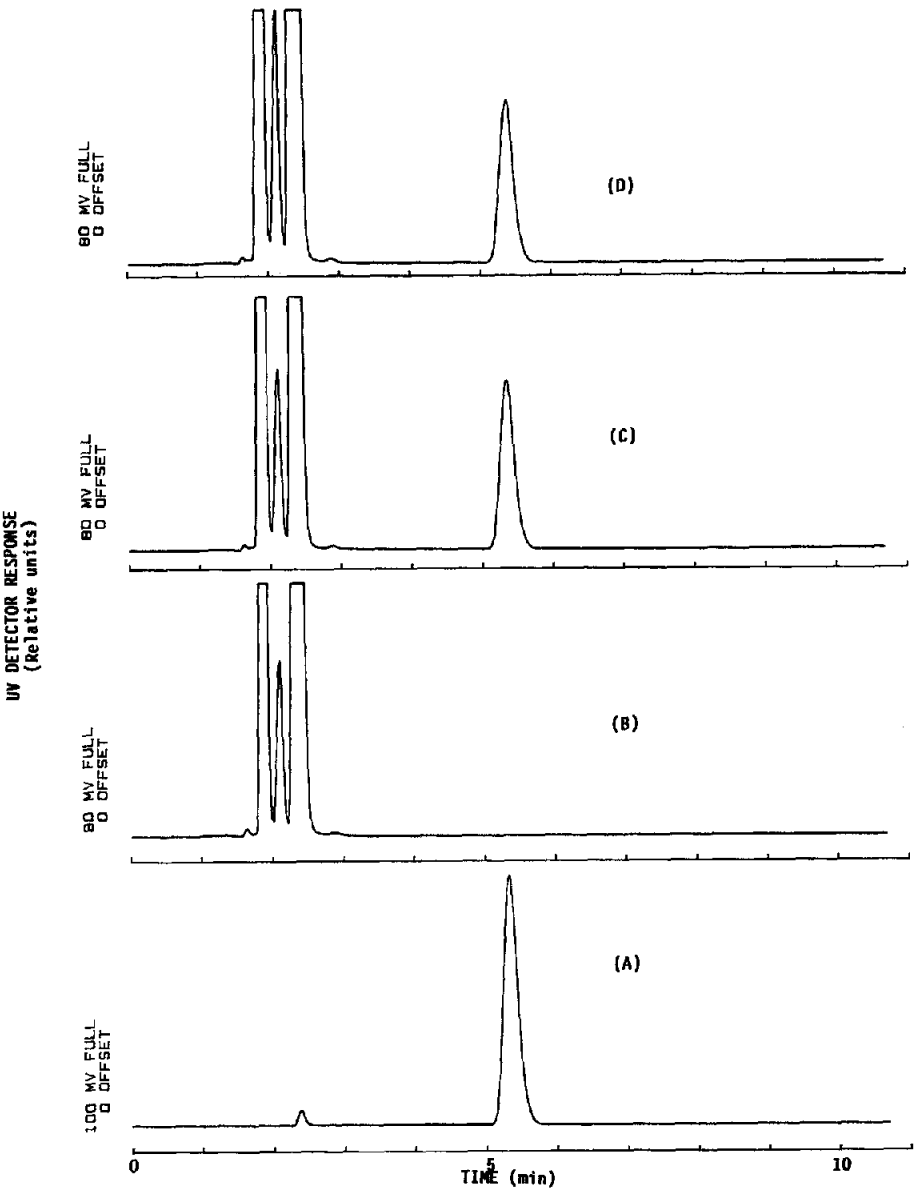

Fig. 2. HPLC chromatograms for synthesized dansyl cyanamide (A), Temposil tablet placebo (B), Temposil tablet (C), and calcium cyanamide bulk material (D).

To evaluate the yield of the derivatization process, calcium cyanamide of known purity was assayed according to the procedure above against the synthesized dansyl cyanamide. HPLC results showed the derivatization process *in situ* to be quantitative (99.9%). The peak purity of dansyl cyanamide was verified by the UV scan of the peak of interest using a diode-array UV detector.

Chromatographic conditions

Fig. 2 shows a typical standard and sample chromatograms obtained using the above procedure. The method proposed here proved suitable to the determination of calcium cyanamide in bulk material and citrated tablet formulation. Since dansyl cyanamide exhibits features of amphoterism as indicated by its ^{1}H NMR in d_6-DMSO, the retention time of dansyl cyanamide was found to be very sensitive to pH as well as the organic modifier in the mobile phase. The mobile phase or the pH was modified to obtain optimum separation. An increase in the acetonitrile concentration decreased the retention time. An increase in pH reduced the retention of dansyl cyanamide. However, it is not recommended to increase the pH of the mobile phase above 7.0.

Linearity, precision, accuracy and sensitivity

Aliquots of calcium cyanamide solution (10–50 mg/100 ml) were taken and derivatized as described under *Derivatization procedure*. Calibration graphs were constructed of peak area *versus* concentration. The results of linear regression analysis were slope 0.0036, intercept 0.5066 and correlation coefficient $r = 0.9999$ ($n = 11$).

The precision of the proposed method was evaluated by ten replicate injections of solution of bulk material (225 μg/ml). For replicate analyses of a sample (50 mg/tablet) were taken to calculate the assay precision in the dosage form. The intra-day assay variation (% R.S.D.) for the bulk material and tablet were 0.20% and 0.67%, respectively. The inter-assay variation was determined by replicate analyses of a 50 mg/tablet of Temposil over a three-day period in two laboratories with the mean inter-assay coefficient of variation (%) calculated to be 1.13%.

The accuracy of the proposed method was examined by preparing tablet placebo samples containing different amounts of calcium cyanamide equivalent to 50, 100 and 150% of label strength. No interference was observed from the tablet excipients. The overall % recovery of calcium cyanamide was 99.43 $\pm$ 1.33%.

The limit of detection is $1 \cdot 10^{-9}$ dansyl cyanamide with a signal-to-noise ratio of 2.0.

Stability

The stability of calcium cyanamide was demonstrated by replicate injections at different time intervals against freshly prepared derivatized standard solution. The dansyl derivative solution was found to be stable for up to 24 h. Calcium cyanamide was stable in either the buffer solution or 0.2 *M* sodium carbonate. Calcium cyanamide in 0.1 *M* hydrochloric acid lost 16% of its potency after 20 h at room temperature.

Forced degradation by UV light, heat, acid, base and oxidation were also performed. The calcium cyanamide solution dissolved in buffer (1 ml) was exposed to UV light for 2 h. Similarly, another 1-ml aliquot of calcium cyanamide solution was heated on a steam bath for 2 h. In addition, 1-ml aliquots were treated with 2 ml of

1 *M* sodium hydroxide, 2 ml of 1 *M* hydrochloric acid and 0.5 ml of 30% hydrogen peroxide. All the degradation solutions were kept at room temperature for 2 h. A 0.5-ml portion of 10 *M* sodium hydroxide was added to the calcium cyanamide–peroxide solution to remove excess peroxide. After degradation, all solutions were diluted with 0.2 *M* sodium carbonate and then derivatized for the HPLC assay. One flask containing calcium cyanamide was set aside as the control. Results of the study showed that calcium cyanamide was stable after UV, acid and base treatment. However, it lost 20% and 100% of its potency after its reaction with heat and hydrogen peroxide, respectively.

Assays

The results of the HPLC analysis indicate that the new method can be used for the quantitation of calcium cyanamide in both bulk material and tablet formulation. Comparison of the results obtained by the HPLC method with the titrimetric assay showed that both sets of the results are comparable. Two lots of bulk material were determined to have an HPLC mean potency value of 92.65% ($n = 10$) *versus* 92.85% potency value by titration. Three lots of Temposil tablets had a mean % label strength of 95.00% ($n = 36$). Titrimetric data for the same three lots yielded a mean % label strength of 94.96%.

CONCLUSIONS

A simple stability-indicating assay for the quantitation of calcium cyanamide has been successfully developed, validated and applied to the bulk material of calcium cyanamide and its tablet formulation. The proposed method is an excellent alternative to the ammonium thiocyanate method for the determination of calcium cyanamide. The assay is specific, separating the drug from the excipients and the degradation products. With minor modifications, the assay could also be used for dissolution studies.

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