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DETERMINATION OF BIURET AND UREA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Biuret was subjected to high-performance liquid chromatography on a reversed-phase column (250 × 4.6 mm; particle diameter 5 μm) with 100 m*M* potassium phosphate buffer, pH 6.7, as the mobile phase and was quantified in a UV-detector set at 199 nm. The calibration curve was linear over the range 0.01–0.4 m*M* biuret. Urea in desalted samples was separated by chromatography on an amino phase (250 × 4.6 mm; particle diameter 5 μm) with acetonitrile–water (9:1, v/v) as the mobile phase and was quantified in a refractive index detector, giving a linear calibration curve for at least 0.1–10 m*M* urea. For lower sensitivity, an UV-detector set at 205 nm could also be used. The determinations were accurate (< 2%), with stable retention times (± 0.2%), and the analytes could be fractionated for further analyses. These methods were successfully applied to biological samples.

INTRODUCTION

The elucidation of a biochemical pathway depends largely on conclusive identification and quantification of the putative intermediates[1], in this case biuret and urea[2]. Biuret can be assayed as a copper complex (the biuret reaction) which is measured in a colorimeter or an atomic absorption spectrophotometer, but the reaction is non-specific, involves many steps and requires large amounts of sample[3]. We required a specific and routine determination of low concentrations (*ca.* 0.01 m*M*) which uses low amounts of sample. Urea, in contrast, can readily be determined by a sensitive enzymatic assay[4] or in a colour test of unknown chemical mechanism[5]. However, these reactions are insufficient to prove the identity of an unknown substance, a process generally regarded as requiring at least three independent methods[6].

We have thus developed simple and sensitive high-performance liquid chromatographic (HPLC) methods to determine these compounds in biological samples.

EXPERIMENTAL

Materials

Biuret and urea, both > 99% pure, were from Fluka (Buchs, Switzerland) and

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their identity was confirmed by mass spectrometry. They were used without further purification.

Potassium dihydrogen phosphate, potassium hydroxide (puriss) and the organic solvents used in mobile phases (HPLC grade) were from Fluka. Water was doubly distilled from and stored in glass apparatus. The packing materials tested for HPLC were Nucleosil (Macherey-Nagel, Düren, F.R.G.), LiChrosorb (Merck, Darmstadt, F.R.G.), Partisil (Whatman, Clifton, NJ, U.S.A.) Spherisorb (Phase Separations, Queensferry, U.K.) and Supelcosil (Supelco, Bellefonte, PA, U.S.A.). Cellulose nitrate membrane filters (Sartorius, Göttingen, F.R.G.) were used.

Sample preparation

Samples from bacterial cultures were centrifuged (23 000 *g* for 20 min at 4°C) or filtered (pore diameter 0.2 μm) before chromatography. Samples from enzyme assays were treated with perchloric acid (final concentration 0.5 *M*) and the precipitated protein was removed by centrifugation (23 000 *g* for 20 min at 4°C). The supernatant was neutralized with 5 *M* potassium hydroxide and the precipitate of potassium perchlorate was removed by centrifugation. Samples were stored at −20°C.

No further treatment was necessary prior to analysis for biuret, but samples for the determination of urea had to be desalted before injection into the low-polarity mobile phase of the HPLC system in order to avoid precipitation of salts in the capillary tubing. Samples containing high levels of urea could be diluted in nine volumes of acetonitrile and the precipitate filtered off (pore diameter 0.2 μm) before injection, whereas all samples could be desalted with AG501-X8(D) mixed bed ion-exchange resin (Bio-Rad, Richmond, CA, U.S.A.). Samples (>3 ml) were allowed to flow slowly through 0.2 g of resin in a Pasteur pipette. The first 2.5 ml were discarded and urea was determined in portions of the remaining eluate, from which the recovery was $>95\%$.

Apparatus and assay conditions

Stainless-steel HPLC columns (40 × 4.6 mm precolumns and 250 × 4.6 mm analytical columns; Knauer, Berlin, F.R.G.) were packed in this laboratory (packing device 80.00, Knauer) according to the manufacturer's instructions; $>25\,000$ theoretical plates per m were observed and found to be adequate for these determinations. Filtered (pore diameter 0.2 μm) mobile phases were degassed by sonic vibration immediately prior to use.

The HPLC system was used isocratically but had a low-pressure mixing chamber for gradients and thus required exhaustive degassing of the mobile phase, which was occasionally sparged with helium (about 2 ml/min) and then drawn through a vacuum degasser (ERC-3310; Erma Optical Works, Tokyo, Japan) before entering the pump (pump module 870, with gradient controller 8800; Du Pont, Wilmington, DE, U.S.A.). The mobile phase passed through a pulse dampener (TM; Ermatech, Bern, Switzerland) and an autosampler with a 20-μl loop (MSI 660; Kontron, Zürich, Switzerland) into the column (an analytical column with a precolumn containing identical stationary phase). Together with the samples separated on the column, it then entered a detector and a programmable fraction collector (Frac 100; Pharmacia, Uppsala, Sweden); residual fluid was transferred to a safe waste past the U-tube of a sink through which water was flowing.

The output from the detector was coupled to the first channel of a slave recorder (Servogor 220; BBC, Baden, Switzerland) and to a computing integrator (SP 4100; Spectra Physics, Santa Clara, CA, U.S.A.). The integrator, detector and fraction collector were interconnected to enable the automatic collection of peaks or fractions of peaks. The second channel of the recorder monitored the pressure in the HPLC system, thus allowing rapid diagnosis of changing retention times due to gas bubbles, leaks or temperature changes. A direct reading flow meter (Phase Separations) was a valuable help in trouble shooting. An electronic noise filter (Knauer) could be mounted between the detector and the recorder when working at high sensitivity.

In the biuret assay the columns had a reversed-phase packing (Nucleosil 5 C18) and were routinely used at room temperature (about 20°C). The mobile phase was 100 m*M* potassium phosphate buffer (pH 6.7) and the flow-rate was 1 ml/min. The detector was a UV-spectrophotometer (Du Pont) set at 199 nm.

Urea was determined after chromatography on an amino phase (Nucleosil 5-NH_2) which was maintained at 0°C by immersing the columns in an ice–water slurry. The mobile phase was acetonitrile–water (9:1, v/v), which was slowly mixed by a magnetically driven stirring bar, and was maintained at 0°C in an ice–water slurry to prevent loss of the organic portion and consequent drift of the baseline. The flow-rate was 1 ml/min. The outlet from the column was thoroughly insulated all the way to the refractive index detector (ERC 7510, Erma) which was internally thermostatted at 30°C and mounted in a polystyrene box. Overnight equilibration of the column and mobile phase was necessary for a stable baseline: when the methanol, in which the columns were stored, had been eluted, the mobile phase was pumped in a closed circuit from the reservoir through the system and back to the reservoir. During equilibration only, the mobile phase was sparged with helium. For lower sensitivity an UV-detector set at 205 nm was used and no overnight equilibration was necessary.

RESULTS AND DISCUSSION

Biuret

Biuret could readily be measured by HPLC (Fig. 1A), capacity ratio 0.65 (retention time 5.1 min). The negative peak was due to dilution of mobile phase by water in the sample, the compound at 4.5 min was putative ammelide (about 0.1 mol%) and the substance at 12.6 min was not identified. The retention time for biuret was constant ($\pm$ 0.2% for ten measurements) as was the peak area ($\pm$ 2% for ten measurements). The peak area (or peak height) was directly proportional to the quantity of analyte in the range 0.01–0.4 m*M* (Fig. 1B). Material from growth medium or enzyme assays did not interfere with the assay. The peak (Fig. 1A, 5.1 min) was confirmed to represent biuret by studying UV spectra of collected fractions. At pH 13, $\lambda_{max.}$ was at 216 nm in agreement with published data[7]: this strong absorption under alkaline conditions was very sensitive to pH and a strong blue shift was observed with increasing pH.

The assay conditions reflect the need for a highly polar mobile phase to enable adequate interaction of analyte and the non-polar stationary phase. The buffer concentration was a compromise between improved separation and wear of the pump

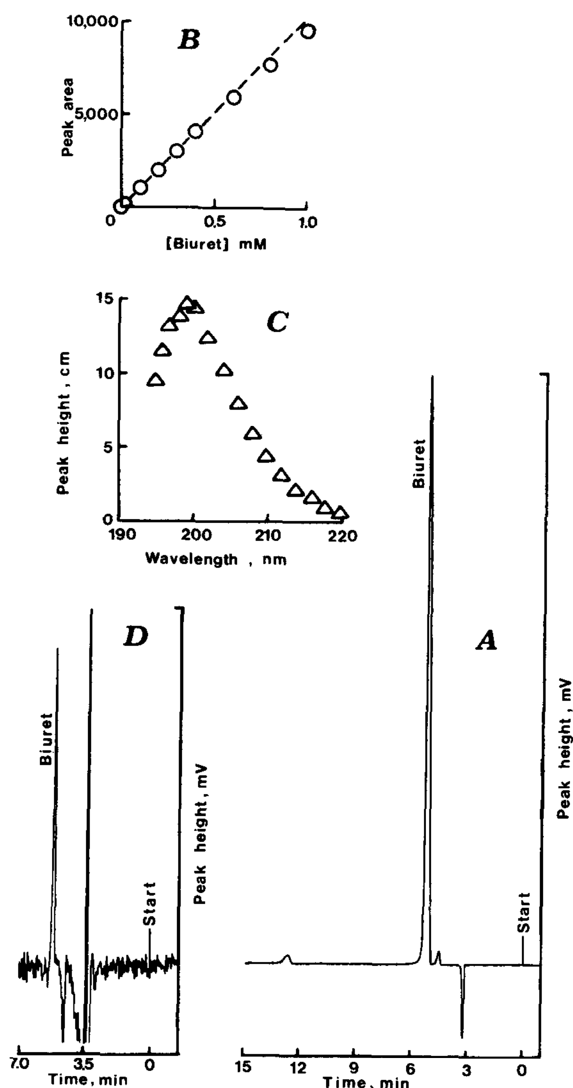

Fig. 1. A, Typical chromatogram of biuret on reversed phase; biuret (10 m*M*) was eluted at 5.1 min. B, Typical calibration curve (peak area in arbitrary units); the extrapolation reveals the departure from linearity. C, UV absorption of a constant concentration of biuret (pH 6.7) in the detector, as a function of wavelength. D, The detection of 0.01 m*M* biuret; the electrical noise filter was installed between the detector and the recorder.

seals. Critical for sensitivity was the wavelength of about 199 nm (Fig. 1C), which allowed a detection limit of about 0.001 m*M*.

Other stationary phases can be used in place of the 5-μm Nucleosil packing and we regularly used a 7-μm packing when no separation from cyanuric acid was needed. The use of LiChrosorb (5 μm) also enabled the separation of biuret and cyanuric acid but with a shorter column life and higher peak asymmetry. Partisil did

not separate these two compounds and Supelcosil (5 μm) separated the compounds only at 0°C, the order of elution being reversed.

Our method for the determination of biuret is specific, sensitive and simple. We have thus achieved our aim of complementing the complex and non-specific versions of the biuret reaction, which require many milligrams of biuret per sample[3].

Urea could not be adequately measured under these conditions. There was a fifty-fold lower sensitivity (than for biuret) and a capacity ratio of 0.20, comparable to other components of growth media (data not shown). Further, at high sensitivity, this portion of the chromatogram could not even be used for material dissolved in distilled water, because of extreme baseline fluctuations (Fig. 1D). There is a report[8] of an urea assay on a reversed-phase column with water as the mobile phase but the sensitivity of the method is apparently low.

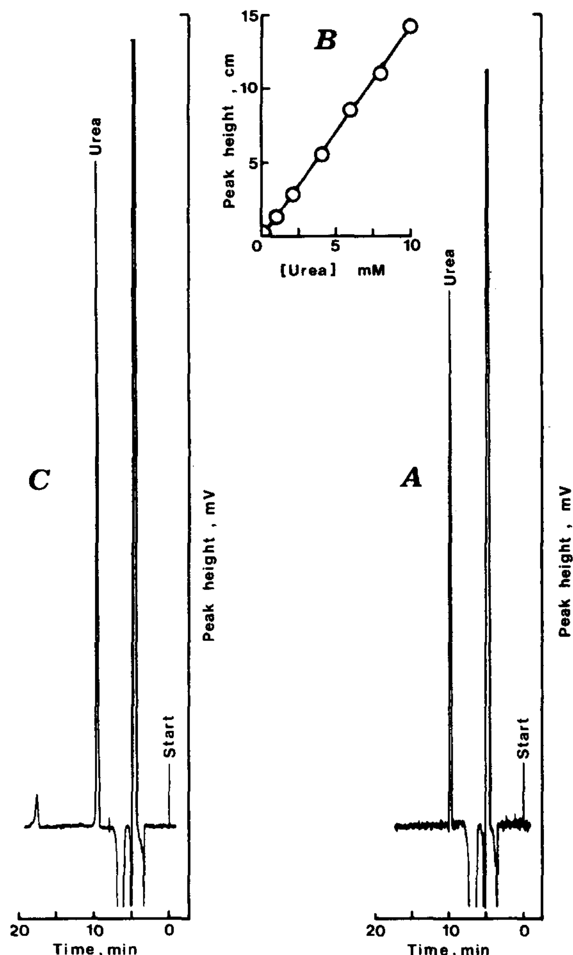

Fig. 2. A, Typical chromatogram of urea on an amino phase obtained with a refractive index detector; urea (1 mM) was eluted at 9.7 min. B, The calibration curve. C, The determination of urea in urine diluted forty-fold; only one other peak was observed (at 17.8 min).

Urea

Urea could readily be determined by chromatography (Fig. 2A). The retention time (9.7 min ± 0.1% for five measurements) and peak height (± 0.3% for five measurements) were constant and the recorder response was proportional to the amount of analyte between 0.1 and 10 m*M* urea (Fig. 2B). No interference from compounds in enzyme assays was observed and urea in urine could easily be measured (Fig. 2C). The extreme response of the refractive index detector to elution of the sample matrix between 3 and 8 min was reproducible for all samples. Material corresponding to the peak at 9.7 min was collected and confirmed to be urea by its reaction with urease.

The choice of mobile and stationary phases was important. Acetonitrile is toxic and we tested acetone–ethyl acetate–water mixtures (*e.g.*, 5:4:1, v/v) which gave partial separation of urea from water with a 5-μm Spherisorb amino phase. The main problem was the loss of volatile components of this mobile phase from the reservoir and the consequent baseline drift in the detector, so we reverted to using acetonitrile–water mixtures. A nitrilo phase (Nucleosil, particle diameter 5 μm) gave no separation of urea from water. The Spherisorb amino phase showed less efficient separation than Nucleosil 7-μm diol or 10-μm amino phases and the best separation was on 5-μm Nucleosil material. When present, biuret was eluted before urea and obscured by the water peak (data not shown).

The method was developed as a technique to help identify urea as the product of a novel reaction (from biuret[2]) and complement existing routine assays. This aim has been achieved and urea can be isolated from complex mixtures by this method. It may even be feasible to apply this method on a routine basis to avoid the corrosive reagents used in the colorimetric assay of urea.

Apparatus

Our Du Pont HPLC system is complex and requires a large array of ancillary equipment for its successful operation. This should not detract from the basic simplicity of the methods described. The biuret determination requires a pump, a detector capable of measuring at 199 nm and a recorder and could be done with our simpler apparatus[9] when the mirror was newly cleaned. The determination of urea requires a pulse-free pump (suitable pumps are commercially available) or a good pulse dampener connected to a conventional pump, a refractive index detector which is internally electronically thermostatted and a recorder; if only low sensitivity is required, the pulse-dampening and the thermostatting are unnecessary and a UV-detector may be used.

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