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Note

Liquid chromatographic determination of urea and ammonia in body fluids using a post-column enzymatic reactor

H. JANSEN, E.G. VAN DER VELDE, U.A.Th. BRINKMAN and R.W. FREI*

Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)

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The determination of urea in serum and in urine is a very important routine test widely used in many clinical laboratories. These measurements are especially important as a means of estimating the nitrogen balance in hospitalized patients who are malnourished [1]. Blood urea concentration is used as an indicator of renal function [1].

In the literature, a wide variety of methods for urea determination can be found which are mostly based on a colour reaction of urea with a reagent or enzymatic hydrolysis of urea by the action of urease followed by some method of detection of the evolved ammonia. A widely used reagent for urea is diacetylmonoxime which forms a yellow chromogen. Enzymatic methods involve a coupled urease—glutamate dehydrogenase method in which the NAD^+ formed is measured by monitoring UV absorption at 340 nm and the urease conductivity method which measures the rate of increasing conductivity as urease converts urea into ammonium and bicarbonate ions. The latter three techniques have been compared recently [1]. Techniques involving a flow-through reactor with immobilized urease include methods in which an ammonia gas electrode is used [2], the evolved ammonium ion is measured in an ion-selective electrode cell [3], the second-derivative spectrophotometry of ammonia is carried out in the gas phase [4] and a method in which an ammonia gas-sensitive semiconductor device is used [5].

Most methods that use the action of urease suffer from positive interference of ammonia. Ways to overcome this problem are (a) the use of a pretreatment procedure to remove ammonia and (b) repeating the experiment without the urease reactor, calculating the urea content by difference. Interference from

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ammonia is not too critical for analysis of serum since ammonia levels are very low as compared to urea levels [6], but it is important in urine, especially during starvation because then about two thirds of urinary nitrogen may be excreted as ammonia and only one third as urea [1].

The present paper describes a method for the simultaneous determination of urea and ammonia without cross-interference that utilizes an ion-pair liquid chromatographic separation with on-line conversion of urea into ammonia followed by fluorescence detection after the addition of *o*-phthalaldehyde (OPA) reagent [7]. A fast and simple pretreatment procedure to reduce interference by amino acids is given. The method was used for the determination of urea and ammonia in urine and for that of urea in serum.

EXPERIMENTAL

Chemicals

Throughout this work, demineralized water, which was purified by filtration in a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used. The eluent was prepared by dissolving 0.005 *M* sodium octylsulphonate (Eastman Kodak, Rochester, NY, U.S.A.) in a 0.05 *M* aqueous potassium phosphate buffer of pH 6.9. The OPA reagent was prepared by adding 0.8 g of OPA (Merck, Darmstadt, F.R.G.) dissolved in 10 ml of ethanol and 1 ml of mercaptoethanol (Janssen, Beerse, Belgium) to a borate buffer, prepared by dissolving 24.7 g of boric acid in 1 l of water, and adjusting the pH to 10.2 with potassium hydroxide. The reagent, which was routinely stored under nitrogen at 4°C when not in use, is stable for at least one week. Test mixtures of urea and ammonia were made by dissolving urea (Merck) and ammonium sulphate (dried overnight at 95°C and stored in a desiccator) in water. Urease (urea amido hydrolyase, EC 3.5.1.5, Sigma, U-2000, St. Louis, MO, U.S.A.) was immobilized on silica (particle diameter 10 μm) as described previously [7].

Apparatus

A schematic diagram of the experimental set-up is given in Fig. 1. The system consists of two pumps, i.e. a reciprocating high-pressure pump (Series 100; Altex, Berkeley, CA, U.S.A.) for the eluent supply and a reciprocating low-pressure pump (Aerograph PCR-1, Varian, Walnut Creek, CA, U.S.A.) for the reagent supply, and two six-port valves, one for the sample introduction (equipped with a 10-μl loop) and one for switching the urease solid-phase reactor (SPR) in and out of the eluent flow. The OPA reactor was a piece of PTFE tubing (0.2 mm I.D.) with a volume of 0.6 ml that was integrated with a mixing T-piece in a cassette (PCR-1 cassette, Varian). The effluent from the reactor was led through a Model 204A fluorescence detector (Perkin-Elmer, Norwalk, CT, U.S.A.) operated at λ_{ex} = 340 nm and λ_{em} = 455 nm. The detector output was registered by a strip chart recorder (BD 8; Kipp & Zonen, Delft, The Netherlands). The analytical column (150 mm × 3 mm I.D.) was home-packed with 5-μm Spherisorb ODS-2 (Phase-Sep, Queensferry, U.K.). A guard column (60 mm × 2 mm I.D.), packed with a C_{18} bonded-phase silica was used to protect the analytical column. The urease-SPR was made by packing a column (60 mm × 3 mm I.D.) with immobilized urease as described

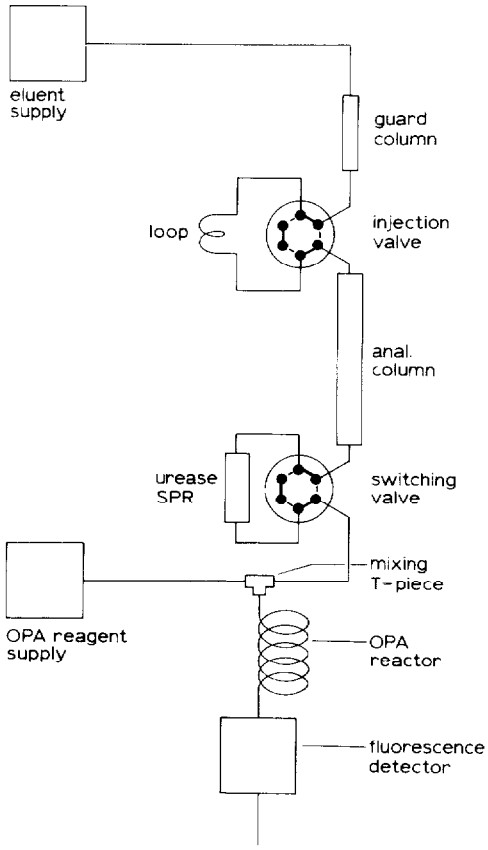

Fig. 1. Schematic diagram of the equipment (for details, see text).

before [7]. The system was operated at room temperature. Eluent and OPA reagent were pumped at 0.5 ml/min each.

Sample pretreatment procedure

In order to reduce the interference of amino acids, an off-line sample pretreatment was developed. First the sample was diluted with Millipore-filtered water, 1000-fold for urine and 100-fold for serum. This was followed by filtration over a Millipore filter (pore width, 0.2 μm). Next, the pH was adjusted to 10—11 with potassium hydroxide. A pretreatment column was prepared by filling a pasteur pipette (5 mm I.D.) with a glass wool plug and a strongly basic anion exchanger (Dowex 1-X2, 50—100 mesh, chloride form; J.T. Baker, Deventer, The Netherlands). The bed height of the anion exchanger was ca. 4 cm. A few millilitres of water were added to the pretreatment column; the water was allowed to drip from the column under gravity flow conditions leaving a wet pretreatment column, ready for use. Next, 2 ml of the diluted sample were added to the column. The first 1.5 ml of eluate were discarded. From the next fraction, 10-μl aliquots were analysed in the high-performance liquid chromatographic (HPLC) system. The pretreatment columns had an internal volume of 0.5—0.6 ml. The 1.5 ml that were discarded guarantee that the water is totally replaced by the sample solution, so that no

dilution of the latter occurs. Each pretreatment column was used only once.

RESULTS AND DISCUSSION

Reaction performance and regeneration procedure

When starting the project, we encountered problems with the urease reactor. After about 8 h, the conversion of urea into ammonia suddenly dropped from 100% to almost 0%. Other reactors behaved similarly. This decline in activity was probably caused by traces of heavy metals, since flushing the urease-SPR for 30 min with 5 m*M* EDTA in 0.05 *M* potassium phosphate buffer (pH 6.9) re-established its full activity. Heavy metals are well known inhibitors of urease and are presumably released by parts of the pumping system. After flushing the pumping system with the same EDTA solution prior to use, we could maintain reactor performance without difficulties. Routinely, the reactor was treated with the EDTA solution once every two weeks. When not in use, the reactor containing the mobile phase was stored at 4°C. The life-time of the reactor, which was almost in daily use, was at least two months.

General analytical data

The total residence times of urea and ammonia were 203 and 414 s, respectively. The retention times on the column were 110 s for urea and 321 s for ammonia. The residence times in the urease-SPR and in the OPA-reactor were measured to be 53 and 40 s, respectively.

Detection limits in aqueous test mixtures at a 3:1 signal-to-noise ratio were found to be ca. 0.3 ng for both urea and ammonia; with the 10-μl injection loop this corresponds to 30 ppb. The repeatability of peak-height measurements was ca. 2% relative S.D. (n = 5) for injections of 11.2 ng of urea and 10.2 ng of ammonia. Linearity of response was observed from the detection limit up to an injected amount of at least 0.5 μg for urea and ammonia. Throughout this range, 100% conversion of urea into ammonia was found.

Determination of urea and ammonia in urine

Injection of 10 μl of a 1000-fold diluted and filtered urine sample onto the C_{18} bonded-silica—octylsulphonate-containing aqueous phosphate buffer system followed by on-line hydrolysis of urea and fluorescence monitoring after OPA derivatization yielded the chromatogram of Fig. 2a. In order to decrease the interferences caused by amino acids which will also react with OPA, the off-line sample pretreatment procedure as described in the experimental section was carried out. This procedure caused a reduction of the peak heights of the interfering peaks by a factor of 5–10. The chromatogram of a cleaned-up urine sample is shown in Fig. 2b. It is easier to see the effect of the pretreatment procedure when chromatography is done without the urease-SPR. Such chromatograms are presented in Fig. 2c and d. Note that the peaks elute 53 s earlier since now no time is spent in the urease-SPR. Without pretreatment, the height of the peaks at 2–2.5 min total residence time (Fig. 2c) that co-elute with urea is about 7% of the height of the urea peak. With pretreatment, they contribute at most 1.5% to the urea peak height (Fig. 2d). Using a longer pretreatment anion-exchange column did not further reduce the interfering peaks. Recoveries in

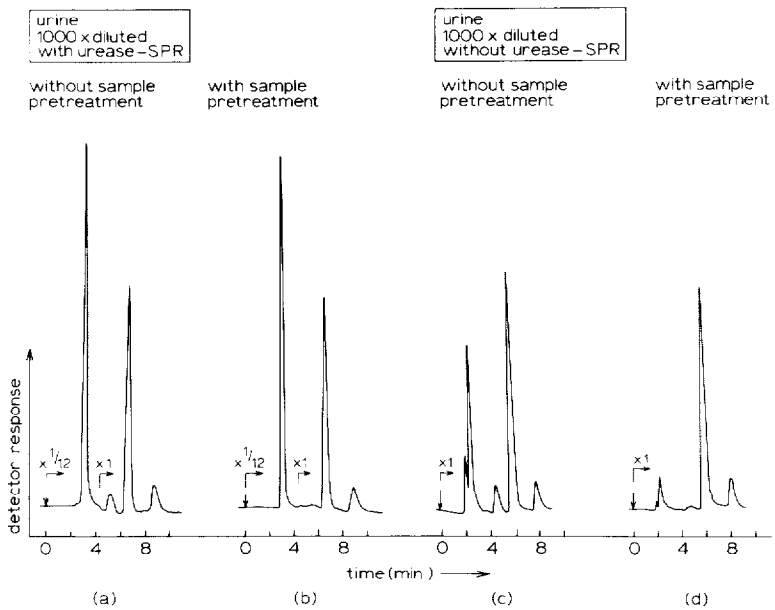

Fig. 2. (a) Chromatogram of a 1000-fold diluted urine sample without pretreatment procedure. Injection volume 10 μl. Column, 150 mm × 3.0 mm I.D., 5-μm Spherisorb ODS-2; eluent, 0.05 *M* potassium phosphate buffer (pH 6.9) with 0.005 *M* sodium octylsulphonate. Urease-SPR, 60 mm × 3.0 mm I.D. stainless-steel column packed with immobilized urease. Flow-rates of eluent and OPA reagent, 0.5 ml/min each. Fluorescence detection, 340 (ex) and 455 (em) nm. Note: × 1/12 means that the actual peak height is twelve times the peak height as indicated in the figure. (b) As Fig. 2a, but with a urine sample cleaned by pretreatment procedure (cf. text). (c) As Fig. 2a, but without urease-SPR. (d) As Fig. 2b, but without urease-SPR.

the pretreatment procedure were measured for test mixtures and spiked urine using different concentrations in the calibration range studied, and were found to be > 99% for urea and 93 ± 0.5% for ammonia. The 7% loss of ammonia is presumably due to partial evaporation of ammonia from the alkaline solution. It can be concluded that the pretreatment procedure is very suitable when urea is to be determined and can be used for the determination of ammonia when making corrections for the loss of ammonia. More accurate determination of ammonia is possible when the pretreatment procedure is omitted, since no interfering peaks from amino acids are observed at the retention time of ammonia. Concentrations of urea in seven urine samples were found to range from 17.9 to 23.5 g/l. Urea concentration in the total urine excreted in a 24-h period was determined to be 18.5 g/l. For ammonia, concentrations ranged from 545 to 660 mg/l with an average value over one day of 613 mg/l. These values agree well with literature data [6].

Determination of urea in serum

Chromatograms of 100-fold diluted and filtered serum samples without and with pretreatment are given in Fig. 3a and b, respectively. Without the urease-SPR, the chromatograms of Fig. 3c and d are obtained. Comparison of Fig. 3b and d reveals that interfering peaks contribute about 10% to the height of the urea peak, even after ion-exchange clean-up. Although a correction factor can

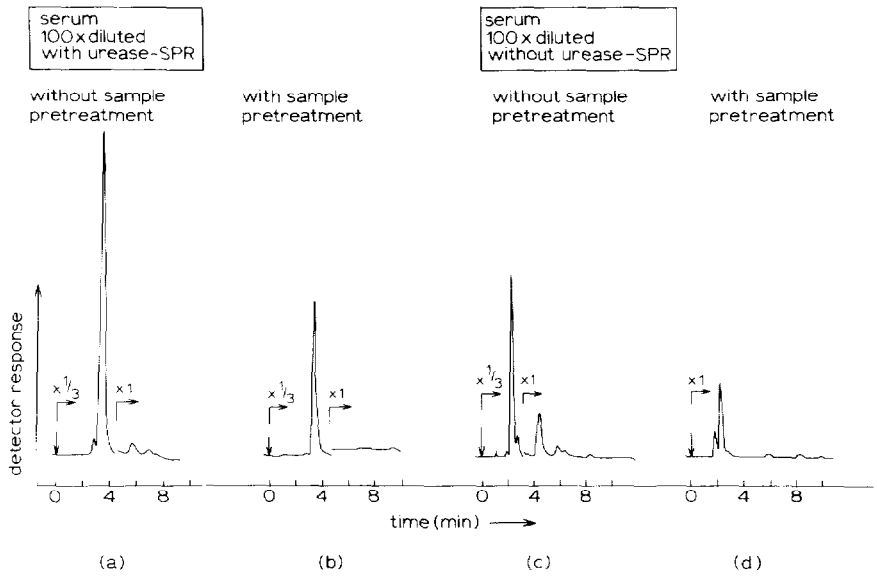

Fig. 3. (a) Chromatogram of a 100-fold diluted serum sample without pretreatment procedure. Conditions as in Fig. 2. (b) As Fig. 3a, but with a serum sample cleaned by the pretreatment procedure. (c) As Fig. 3a, but without urease-SPR. (d) As Fig. 3b, but without urease-SPR.

be introduced to compensate for this error, a loss of accuracy results. Concentrations of urea in serum were found to be about 230 mg/l. A more accurate determination of urea is possible by measuring peak heights with and without urease-SPR; an obvious disadvantage of this procedure is that twice as many runs are required.

In the diluted samples, ammonia concentrations were below the detection limit of 30 μg/l, which is in agreement with literature data [6].

CONCLUSIONS

The method described offers an attractive way for analysis of urea and ammonium in urine and serum. Only a simple sample pretreatment procedure is needed. The urease-SPR can easily be regenerated when its activity has dropped, because of the action of heavy metals, by flushing it with a phosphate buffer containing EDTA. When routinely regenerated every two weeks, its activity lasts for at least two months when in daily use. It should, however, be remembered that the urine and serum samples were considerably diluted prior to analysis because of the highly sensitive detection. This dilution results in cleaner samples and will have a positive effect on reactor life-time. Another important advantage related to the dilution is that problems of clogging of frits and the analytical column are circumvented.

The method is very suitable for the simultaneous determination of urea and ammonia in urine, without cross-interference. The 1000-fold dilution permits the quantitation of urea and ammonia in the linear range of each of their calibration plots even though the urea concentration is about 30 times higher than the ammonia concentration. For an accurate urea determination, the

sample pretreatment procedure is to be used, whereas for best results in ammonia determinations, that procedure has to be omitted.

The determination of urea in serum samples can conveniently be carried out by the present method, although the necessity to apply a correction factor may introduce some errors. However, in clinical analysis it is not always necessary to have highly accurate data. In such cases, the method can certainly be recommended since it has the additional advantage that the required serum sample is only very small.

Finally, the method including the sample pretreatment procedure has an obvious potential for automation, which makes it even more attractive for routine analysis of large series of samples.

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