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Note

Rapid determination of plasma urea by gas chromatography*

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Urea, NH_2-CO-NH_2, is the chief end product of protein (ammonia) metabolism in mammals. It is formed almost exclusively in the liver from the amino groups of amino acids, and upon entering the blood, most of it is rapidly excreted into the urine. The urea plasma level may provide a rapid and efficient method for assessing N availability and protein quality or for studying the enzyme activities of urease, arginase, etc. Physiological abnormalities such as hepatic disease or renal failures may affect the blood urea concentration. As a source of non-protein nitrogen, urea is fed extensively to the ruminant animals as a protein supplement[1].

Two basic assay methods[2] have been used for plasma urea, *viz.* the colorimetric method (diacetyl monoxime) and the urease method, for the subsequent photometric determination of hydrolyzed NH_3. Gas chromatographic (GC) procedures[3] for determining urea as trifluoroacetyl derivative in animal tissues using electron capture detection are also available. The methods, however, require laborious sample preparation and cleanup and large quantities of material (10 g).

This report describes a rapid and sensitive GC method using N detection for assaying trifluoroacetylurea from μl quantities of blood. The advantages of using the GC-N detection system for determining amino acids[4–6], peptides[7], vitamins[8], and amino sugars[9] have been documented previously.

EXPERIMENTAL

The gas chromatograph used in this study was a Tracor MT-220, four-column oven, equipped with a Coulson electrolytic conductivity detector. A 6 in. × 1/4 in. I.D. U-glass column packed with 0.325 w/w % EGA on 80–100 mesh HT Chromosorb W AW was used for the separation of urea from other organic amino compounds present in the sample.

Solutions (5 m*M*) of urea (Baker Analyzed reagent) were prepared in 0.1 *N* HCl. Nitrobenzene (0.5 m*M*) in hexane was used as internal standard. Samples of animal and human plasma (5 μl each) or of standard urea solution (10 μl) were introduced into 0.5 ml cone-shaped micro-vials for direct acylation[10] without prior cleanup. The preparation of the acyl derivative included: (1) drying the samples at 70 under a stream of dry nitrogen, (2) adding 10 μl of internal standard, (3) adding

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100 μl of dichloromethane–trifluoroacetic anhydride (3:1), (4) screwing vial with PTFE-lined cap tightly, (5) high-speed mechanical mixing (against the inner edge of the Neoprene head) for 30 sec, (6) ultrasonic mixing for 1 min, and (7) acylation at 25° for 30 min. The GC instrumental setting and parameters are shown in Table I.

TABLE I

GC CONDITIONS FOR THE SEPARATION OF PLASMA UREA

Column	0.325 w/w% EGA* on 80–100 mesh HT Chromosorb W AW
Column temperature, °C	100–200 (10°/min, programmed)
Inlet temperature, °C	210
Helium flow-rate, ml/min	
Carrier	60
Pyrolyzer	10
Hydrogen flow-rate, ml/min	
Pyrolyzer	50
Pyrolyzer temperature, °C	820
Chart speed, in./min	0.5

* EGA = Ethylene glycol adipate.

The quantitative aspects of urea analysis were checked by the procedure of relative response factor by internal standard method. Five independent measurements were made and the relative peak height ratio was calculated as shown in Table II. The internal standard, nitrobenzene, was chosen particularly because it is stable, foreign to the test samples, and of close retention to urea.

TABLE II

DETERMINATION OF UREA RELATIVE YIELD BY PEAK HEIGHT RATIO OF UREA *vs.* INTERNAL STANDARD (IS)

Trial	*Derivatization**	*Peak ratio (urea IS)*			
		3-μl sample load	*4-μl sample load*	*5-μl sample load*	*Average**
1	25°/30 min	3.28	3.30	3.29	3.29
2	25°/60 min	3.30	3.31	3.32	3.31
3	25°/18 h	3.31	3.30	3.31	3.31
4	25°/24 h	3.29	3.30	3.31	3.30
5	25°/72 h	3.29	3.29	3.28	3.29
					3.30 ± 0.01

* Higher temperature derivatization, *e.g.*, 100°/10 min or 150°/5 min, would decompose urea and reduce the relative yield.

Initial studies, including the analyses of blanks and reagents, were tested prior to sample analysis. The region of relative retention time or temperature for internal standard was carefully checked for interference, which might possibly be overlapping with the internal standard. The chromatograms of the reagents (two-fold of test sample load) indicated that no unknown peak should show at the retention of urea or of the internal standard.

Experiments on recovery of urea from chicken plasma were designed by adding increasing amounts of urea solutions (0.3 to 1.5 μg) to the plasma samples (5 μl) and by measuring the increases in response (internal standard load was constant, 10 μl) due to these additions as compared to the response of samples with zero addition. The percent recovery of each plasma fortified with authentic urea in the presence of other organic amino substances was calculated.

RESULTS AND DISCUSSION

Fig. 1 (a–f) shows the typical GC chromatograms of separation of urea from other components including internal standard on the EGA column. A complete run is about 10 min. The amount of urea present in the plasma as shown in the chromato-

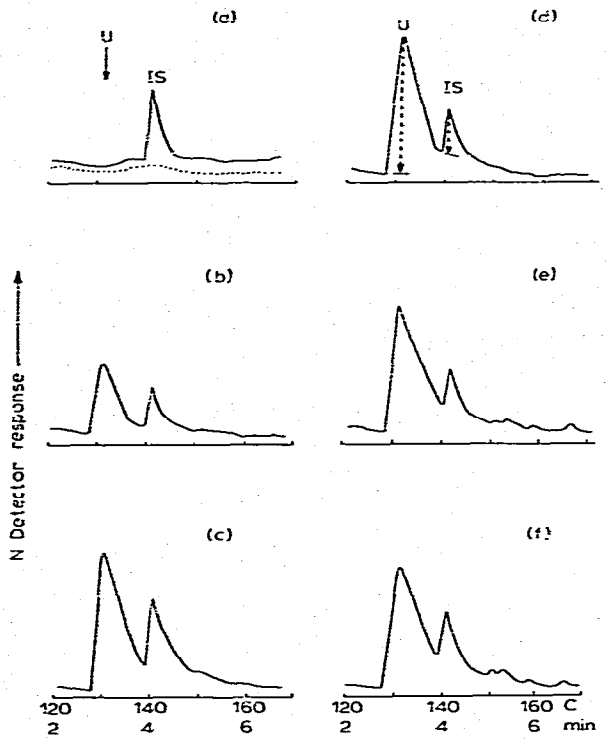

Fig. 1. Gas chromatograms showing the elution, the relative retention, and the relative peak response of urea (U) vs. internal standard (IS) in animal plasma of (a) chicken, (b) rat, (c) cow, (e) pig and (f) child. The GC curve (d) indicates the standard mixture (80 ng urea) and the calibration for peak height by the internal standard (nitrobenzene) method. -----, Reagent.

grams was in the sub-nanomole range. Quantitation of urea can be obtained by measuring the response ratio (urea)/(internal standard) as indicated in Fig. 1d. This, in turn, can be calibrated against the response ratio of a standard mixture. An internal standard method permits correction for volatilization of solvent or dilution of final

derivative mixture and losses during sample manipulation. Quantitative analysis can be readily achieved by using the following formula:

$$\text{mg urea per 100 ml plasma} = \frac{\text{peak ratio in sample}}{\text{peak ratio in calibration mix}} \times F$$

where

$$F = \frac{\mu\text{g std. urea in calibration mix}}{\mu\text{l plasma sample}} \times 100 \text{ or } \left(\frac{1.5}{5} \times 100\right)$$

It should be emphasized that no detectable urea was shown in the GC curves of chicken plasma. Admittedly, one cannot determine urea in the plasma of birds owing to the fact that certain enzymes are missing in the liver for arginine (urea cycle) synthesis. The amino acid arginine, therefore, becomes essential in the poultry diet.

The data in Table II show the relative yield of five independent trials for the (urea)/(internal standard) ratio. Nitrobenzene as internal standard, however, was not being derivatized. These data reflect the basis for reproducibility and quantitation when the internal standard method is used. Solutions of derivatized urea are also relatively stable. The average peak height ratio showed a standard deviation of ± 0.01. This precision is satisfactory for its applications. The high sensitivity and the speed of the procedure provide a significant advantage over classic methods.

Any change in column performance, operating conditions, or concentration of internal standard would affect the value of relative yield. This change became insignificant at the moment when internal standard was introduced simultaneously into the calibration mixture or sample vials.

Table III shows the actual levels of urea found in test plasma obtained from

TABLE III

UREA IN PLASMA SAMPLES

Source	*Sample*	*Urea (mg%)*
Chicken	1	—
	2	—
Rat	1	19.81
	2	19.54
Pig	1	38.36
	2	43.36
Child	1	28.45
	2	27.72
Cow	1	19.17
	2	14.20
		13–58*

* Literature blood value (ref. 11).

normal rats, pigs, cows, and children. The urea content varies from 14 to 43 mg per 100 ml of plasma.

The data in Table IV show the recovery of urea added to the chicken plasma. Per cent recovery was calculated on the mg% basis. With the fortification of 0.3–1.5 μg per vial into the chicken plasma, the minimum recovery was 98%. The spread

TABLE IV

RECOVERY OF UREA FROM PLASMA

Sample No.	*Chicken plasma 5 µl*	*Known level (mg%)*	*Amount added* (µg per vial)*	*Amount found** (mg%)*	*% recovered*
1	Control	0	0	0	--
2	Fortified 1	0	0.30	6.10	102
3	Fortified 2	0	0.60	12.01	100
4	Fortified 3	0	0.90	17.80	98
5	Fortified 4	0	1.20	23.80	99
6	Fortified 5	0	1.50	29.50	98
					98–102

* Solutions (5 m*M*) of urea standard.
** Average of two injections.

of per cent recovery on all samples ranging from 98 to 102% was considered satisfactory for many applications. The accuracy of the method should be comparable to this precision if the sample analyses are performed by other analysts.

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