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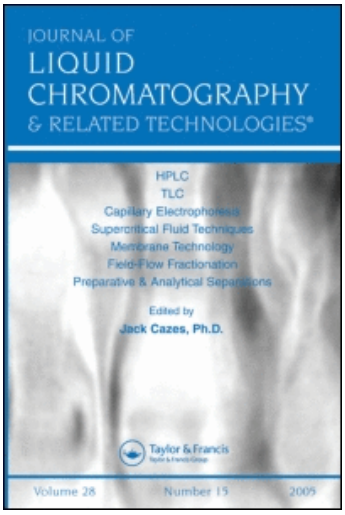

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DIRECT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF GLYCINE BETAINE AND ITS METABOLITE, *N,N*-DIMETHYLGLYCINE, IN PHARMACOKINETIC STUDIES

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ABSTRACT

A simple and direct, without derivatization, method for the routine analysis of glycine betaine and its metabolite *N*,*N*-dimethylglycine in body fluids, serum or urine, is described. Low wavelength ultraviolet detection is achieved at 198 nm using 20 mmol/L KH_2PO_4 at a flow rate of 1.2 mL/min as a mobile phase and a Nucleosil 100-10 SA (10 μm, 250 $\times$ 4.6 mm) as an analytical column. The limit of detection is 7.5 ng for both compounds and the limit of quantitation is 25 ng when 50 μL is injected onto the column. A rectilinear relationship is observed up to 120 ng/μL. Method validation performed by day-to-day ($n = 8$) and within-day ($n = 8$) precision assays gave relative standard deviation values in the range of 5.1–10.5% for glycine betaine and 3.6–9.0% for *N*,*N*-dimethylglycine. 7-Methylxanthine is used as

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the internal standard at a concentration of 0.2 ng/μL. Analysis time is approximately 6 min. The method developed is applied to the analysis of glycine betaine in a commercial pharmaceutical preparation with high accuracy and precision results, as well as to the monitoring of glycine betaine and its metabolite *N*,*N*-dimethylglycine levels in body fluids, serum and urine, as their levels are indicative of renal disorder. Mean recovery of glycine betaine in the pharmaceutical formulation, assayed at different concentration levels, is 102.4% Percentage recovery values of analytes in spiked serum samples range from 90.9 to 106.6 % for *N*,*N*-dimethylglycine and from 95.6 to 100.3% for glycine betaine, while the respective values in spiked urine samples are in the range of 93.6–106.2% and 100.9–103.9%. After the appropriate dilution of biological fluid samples, any matrix interference is eliminated. The method appears to be suitable for monitoring glycine betaine and its metabolite in body fluid, especially in urine of patients undergoing therapy with betaine.

INTRODUCTION

Glycine betaine is a naturally occurring quaternary ammonium compound that functions physiologically as an osmolyte. The function of osmolytes is one of the different strategies that use living cells to compensate in hostile environments such as the occurrence of osmotic stress. The most common response is to accumulate high concentrations, sometimes more than 1 *M*, of one or more solutes, usually polyols or zwitterions, that stabilize macromolecules against denaturation (1,2).

Glycine betaine has an increasingly interesting role as an osmolyte in human and other mammalian systems organs, especially in the kidney. Sodium-dependent transport systems are present in mammalian kidney and these are inducible by osmotic stress (3,4).

As glycine betaine is a common constituent of plant tissues, including many food plants, it is expected to be a common component of a normal human diet. Its blood levels are homeostatically controlled. Whatever the mechanism, the presence of abnormal levels of a major renal osmolyte in the urine of diabetic patients indicates some form of abnormal renal function. Further investigation is required to prove whether glycine betaine excretion can predict later development of clinical renal disease. Normal human plasma concentrations of the renal osmolyte glycine betaine are 20–70 μmol/L and are higher in adult males than in females. These concentrations are lower in renal disease (median 28 μmol/L) and normal in diabetes. Urinary excretion shows no sex difference and is frequently

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elevated both in renal disease and in diabetes. Median values (mmol/mol creatinine) are: normal, 6.2; renal, 12.3; and diabetes, 39.7 (1,5).

Betaine's important role in medicine is due to its function as an alternative homocysteine-methylating agent performing as a substrate for betaine homocysteine methyltransferase. Treatment with betaine has been tested and proved to lower homocysteine levels and increase methionine levels in patients with homocystinuria, a disorder caused by a genetic defect in the transsulfuration pathway of methionine's conversion to cysteine (deficiency of cystathionine β-synthase) or by a defective biosynthesis of the essential cofactors of the remethylation pathway of homocysteine to methionine. Thus, betaine is a constituent at various doses, in clinical trials, for treating homocystinuria. *N*,*N*-Dimethylglycine (*N*,*N*-DMG) originates as a betaine metabolite during homocysteine methylation in blood and urine (6).

Several methods for determination of glycine betaine have been described in the literature, including thin-layer chromatography, electrophoresis, gas chromatography, and precipitation with reineckate or triiodide. A number of studies using high-performance liquid chromatography (HPLC) separations have also been reported. Most of them determine betaine as *p*-bromophenacyl, *p*-nitrobenzyl, or methyl ester derivatives. Solid-phase extraction for separation of betaine and *N*,*N*-DMG was reported after deamination of amino acids by van Slyke method (3–12).

The present paper provides a simple, direct, without derivatization, rapid, and sensitive method for monitoring urine and serum levels of glycine betaine and its metabolite *N*,*N*-DMG.

EXPERIMENTAL

Chemicals

Glycine betaine, *N*,*N*-dimethylglycine, glycine, and 7-methylxanthine were purchased from Sigma (St. Louis, MO, USA). KH_2PO_4 was pro analysis grade, from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were obtained from Riedel-de-Haen AG (Seelze, Germany). Bis-deionized water was used throughout the analysis.

HPLC Instrumentation

An SSI 222 D pump (SSI, State College, PA, USA) was used to deliver the mobile phase to the analytical column, Nucleosil 100-10 SA (10 μm, 250 $\times$ 4.6 mm; Macherey-Nagel GmbH, Düren, Germany). Sample injection was performed via a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 50 μL loop.

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A SSI 500 ultraviolet-visible (UV-Vis) detector working at a wavelength of 198 nm and sensitivity setting of 0.002 absorbance unit, full scale (AUFS) was used for detection. An HP 3396 series II integrator (Hewlett-Packard, Avondale, PA, USA) was used for quantitative determination of eluted peaks. A glass vacuum-filtration apparatus obtained from Alltech Associates was used for the filtration of the mobile phase, with 0.2-μm membrane filters (Schleicher and Schuell, Dassel, Germany).

Degassing of solvents was achieved by sonication in a 460/H ultrasonic bath (Transonic, Elma, Germany) before use. A Glass-col, Terre Haute 47802 small vortexer and a model Z 230 centrifuge (B. Hermle, Gosheim, Germany) were used for the sample pretreatment.

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Chromatographic Conditions

Aqueous stock solutions of glycine betaine, *N*,*N*-DMG, and glycine, at concentrations of 70.0, 100.0, and 120.0 ng/μL, were prepared and stored refrigerated at 4°C. These solutions were found to be stable throughout the experimental analyses. Working aqueous solutions were prepared from stock solutions at concentrations of 0.05, 0.10, 0.25, 0.50, 1.0, 2.0, 3.0, 5.0, 8.0, 15.0, 20.0, 30.0, and 50.0 ng/μL. 7-Methylxanthine was used as an internal standard at a concentration of 0.2 ng/μL.

The mobile phase of KH_2PO_4 was chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution. Inlet pressure observed with the eluent system, at a flow rate 1.2 mL/min, was 775 psi.

RESULTS AND DISCUSSION

Analysis Time

Glycine betaine, *N*,*N*-DMG, and glycine are eluted within approximately 6 min. The chromatogram illustrated in Figure 1 is obtained under the experimental conditions developed in the present assay. Resolution factors were found to be satisfactory, indicating sufficient separation: 1.24 (glycine and *N*,*N*-DMG), 1.63 (*N*,*N*-DMG and glycine betaine), and 1.02 (glycine betaine and internal standard).

Linearity Range

Calibration of the method was performed by injection of standards covering the entire working range. The sensitivity setting of the UV-VIS detector was adjusted at 0.002 AUFS. Each sample was injected six times.

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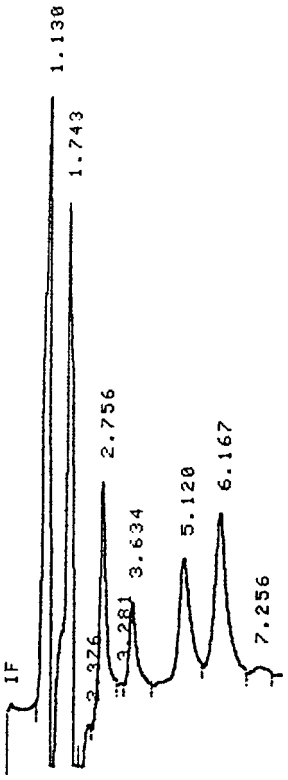

Figure 1. High performance liquid chromatogram of glycine (2.756 min), *N*,*N*-DMG (3.634 min), and glycine betaine (5.120 min) with 7-methylxanthine (6.167 min) as internal standard. Chromatographic conditions are described in text.

Linear correlation between absolute injected amount or concentration and peak area ratio with 7-methylxanthine as the internal standard at a concentration of 0.2 ng/μL was observed. The results of the statistical treatment of calibration data are summarized in Table 1.

The minimum detectable concentration LOD was defined as a peak height that produces 3 times the level of baseline noise at 0.0005 AUFS. The LOQ was the lowest concentration of calibration standards with acceptable precision and accuracy.

Method Validation: Accuracy, Precision, and Stability

The precision of the method based on within-day repeatability was determined by replicate injections ($n = 8$) of four standard solutions covering different

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Table 1. Calibration Data for Glycine, *N*,*N*-Dimethylglycine, and Glycine Betaine Determination with 7-Methylxanthine as Internal Standard

Compound	Regression Equation (a, x = ng/μL; b, x = ng)	Correlation Coefficient	LOD (ng)	LOQ (ng)	Upper Limit (ng/μL)
Glycine	$y_a = (0.02995 \pm 0.00229)x + (0.04338 \pm 0.00185)$ $y_b = (0.00060 \pm 4.58\times10^{-5})x + (0.04338 \pm 0.00185)$	0.9913	2.5	10.0	70.0
N,*N*-DMG	$y_a = (0.17127 \pm 0.00623)x + (0.04527 \pm 0.00171)$ $y_b = (0.003425 \pm 5.14\times10^{-4})x + (0.04527 \pm 0.00171)$	0.9947	7.5	25	120
Glycine betaine	$y_a = (0.42401 \pm 0.03401)x + (0.03661 \pm 0.00324)$ $y_b = (0.00848 \pm 3.78\times10^{-5})x + (0.03661 \pm 0.00324)$	0.9954	7.5	25	120

concentration level, low, medium, and high, where peak areas were measured in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations at different values. Results are shown in Table 2.

The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of 8 consecutive days. Reproducibility results are illustrated in Table 2.

Accuracy was determined by replicate analysis of four different levels (1.0, 5.0,10.0, and 20.0 ng/μL) and calculation of the recoveries of actually found versus spiked values.

The stability of glycine betaine and metabolite solutions was verified by storing sample solutions, refrigerated, for 1 month. Concentrations were measured periodically (1, 2, 3, and 4 weeks).

Application to Real Samples

To validate the assay method, it was applied to analysis of real samples: pharmaceutical preparations and body fluids.

Pharmaceutical Preparations

The only available commercial pharmaceutical preparation containing betaine was a tablet formulation under the trade name Kloref containing 1.035 g of betaine monohydrate as stated on the label.

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Table 2. Day-to-Day (over a Period of 8 Consecutive Days) and Within-Day ($n = 8$) Precison and Accuracy Study for Determination of Glycine Betaine, *N*,*N*-Dimethylglycine, and Glycine

	Within-Day			Inter-Day		
Added (ng)	Found[a] (ng)	RSD[b]	Recovery (%)	Found[a] (ng)	RSD	Recovery (%)
Glycine						
50.00	50.05 ± 3.24	6.5	100.1	49.0 ± 3.27	6.7	98.0
250.00	261.96 ± 15.87	6.1	104.8	232.67 ± 20.78	8.9	93.1
500.00	480.33 ± 47.50	9.9	96.1	530.35 ± 17.93	3.4	106.1
1000.00	1042.33 ± 52.32	5.0	104.2	973.0 ± 44.76	4.6	97.3
N,*N*-DMG						
50.00	50.2 ± 4.13	8.23	100.4	48.53 ± 14.53	7.0	97.1
250.00	246.2 ± 22.21	9.02	98.48	246.2 ± 10.02	4.0	98.5
500.00	497.2 ± 35.5	7.14	99.44	497.2 ± 17.80	3.6	99.4
1000.00	1004.53 ± 65.29	6.50	100.45	1073.8 ± 45.42	4.2	107.4
Glycine betaine						
50.00	47.07 ± 4.73	10.05	94.14	55.05 ± 4.51	8.2	110.1
250.00	254.0 ± 17.96	7.07	101.6	262.3 ± 16.03	6.1	104.9
500.00	492.15 ± 46.71	9.49	98.43	503.8 ± 40.46	8.0	100.8
1000.00	966.21 ± 72.87	10.54	96.62	997.1 ± 50.85	5.1	99.7

[a]Values are mean ± SD.
[b]RSD, relative standard deviation.

Tablets were dissolved in bis-deionized water and sonicated for 10 min to enhance dissolution rate. A stock solution of 897.0 ng/μL betaine was initially prepared, from which dilute solutions of 8.97, 13.45, and 17.94 ng/μL were prepared after subsequent dilutions. Internal standard solution was added to the final solution. This treatment was followed for 5 different tablets.

Figure 2 shows the chromatogram obtained using the conditions described in the text. Results are tabulated in Table 3.

Human Blood Serum

Pooled human blood serum was diluted 300 times after protein precipitation with CH_3CN at concentration levels of 1.0, 2.0, 3.0, 5.0, and 10.0 ng/μL. Internal standard solution was added to the final solution ready for measurement. The calibration curves were constructed from linear regression analysis of the peak area ratios versus the nominal concentrations of the calibration standards. Correlation coefficients were $r = 0.9935$ for *N*,*N*-DMG and $r = 0.9930$ for glycine betaine (Table 4).

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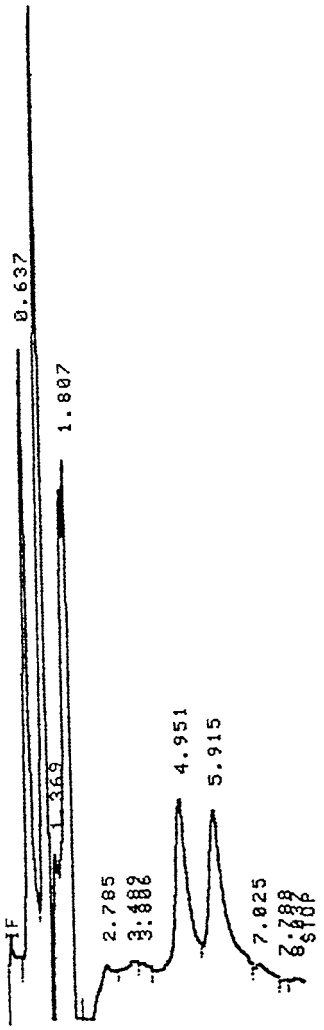

Figure 2. High performance liquid chromatogram of analysis of glycine betaine (4.951 min) in a pharmaceutical preparation (tablet) with 7-methylxanthine (5.915 min) as internal standard. Chromatographic conditions are described in the text.

The concentrations of glycine betaine and metabolite in serum samples were calculated by application of a standard addition technique and found to be 3.45 and 14.4 μmol/L for glycine betaine and its metabolite, respectively.

The high-performance liquid chromatogram of glycine betaine and its metabolite in human blood serum is shown in Figure 3. No interference from endogenous compounds from the sample matrix was noticed.

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Table 3. Results of Glycine Betaine Analysis in a Pharmaceutical Product

	Added (ng)	Found (ng)	SD (ng)	RSD (%)	Recovery (%)
Tablet 1					
	449.00	467.00	36.61	7.8	104.0
	673.00	701.43	60.81	8.7	104.2
	897.00	973.20	89.34	9.2	108.5
Tablet 2					
	449.00	485.29	45.76	9.4	108.1
	673.00	725.86	46.89	6.5	107.8
	897.00	891.23	70.14	7.9	99.3
Tablet 3					
	449.00	445.50	43.36	9.7	99.2
	673.00	785.57	59.72	7.6	106.7
	897.00	872.96	30.99	3.6	97.3
Tablet 4					
	449.00	439.57	27.25	6.2	97.9
	673.00	578.78	0.78	0.2	96.0
	897.00	912.67	10.22	1.1	101.7
Tablet 5					
	449.00	465.03	31.39	6.8	103.6
	673.00	702.00	54.62	7.8	104.3
	897.00	831.43	40.91	4.9	92.7
Labeled amount (g)[a]					
	1.035	1.060[b]	0.096	9.1	102.4

[a]Betaine monohydrate.
[b]Mean value: $n = 5$ tablets $\times$ 3 concentrations $\times$ 6 measurements.

Serum samples containing different concentrations of glycine betaine and its metabolite were found to be stable for at least 1 month without significant decomposition of the analytes. A set of quality control samples were allowed to thaw and then were assayed. The results were compared to the calibration data. These samples were subject to three freeze-thaw cycles and then assayed to evaluate stability of analytes in serum. Long-term stability was studied by assaying samples that had been stored for 1 month.

Urine

Pooled urine samples were simply diluted 1:5000 to eliminate matrix interference, which otherwise would be significant. In this way, any interference from endogenous compounds from the sample matrix was eliminated, allowing the determination of analytes, as shown in the urine sample chromatogram in Figure 4.

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Table 4. Calibration Data for Glycine Betaine and *N*,*N*-Dimethylglycine Determination in Body Fluids with 7-Methylxanthine as Internal Standard

	Regression Equations	Correlation Coefficient
Blood serum		
N,N-DMG	$y = (0.19695 \pm 0.02249)x + (0.00097 \pm 4.8 \times 10^{-5})$ (x = ng/μL)	0.9935
	$y = (0.00788 \pm 0.01847)x + (0.00097 \pm 4.8 \times 10^{-5})$ (x = ng)	
Glycine betaine	$y = (0.44860 \pm 0.00640)x + (0.000604 \pm 1.68 \times 10^{-5})$ (x = ng/μL)	0.9930
	$y = (0.00897 \pm 0.00013)x + (0.000604 \pm 1.68 \times 10^{-5})$ (x = ng)	
Urine		
N,N-DMG	$y = (0.19793 \pm 0.00190)x + (0.00410 \pm 0.00030)$ (x = ng/μL)	0.9953
	$y = (0.00396 \pm 0.00956)x + (0.00410 \pm 0.00030)$ (x = ng)	
Glycine betaine	$y = (0.44348 \pm 0.00382)x + (0.00791 \pm 0.00028)$ (x = ng/μL)	0.9807
	$y = (0.00887 \pm 7.66 \times 10^{-5})x + (0.00791 \pm 0.00028)$ (x = ng)	

Standard addition technique was applied by adding analytes, at five different amount, i.e., 1.0, 2.0, 3.0, 5.0, and 10.0 ng/μL. Internal standard solution was added to the final solution ready for measurement. The calibration curves were constructed from linear regression analysis of the peak area ratios versus the nominal concentrations of the calibration standards.

The concentrations of glycine betaine and metabolite in serum samples were calculated by extrapolating the calibration curve and found to be 762.2 μmol/L equivalent to 3.52 mmol/mol creatinine for glycine betaine and 1005.53 μmol/L equivalent to 4.64 mmol/mol creatinine for its metabolite.

The creatinine level in a pooled urine sample was measured by Jaffe colorimetry in a state hospital biochemical laboratory and was found to be 244.62 mg/dL. This value was used to express urine levels of glycine betaine and its metabolite as mmol/per mol creatinine units.

Biological fluids and urine, most of all, contain complex mixtures of which betaine and its metabolite are minor constituents. During our preliminary trials, we aimed to isolate analytes by solid-phase extraction after or without van Slyke deamination of amino acids. Unfortunately, the matrix elimination was poor, and analyte recoveries were insufficient. Development of the appropriate protocol was hard because of betaine's particular properties as a zwitterion. For these reasons, we

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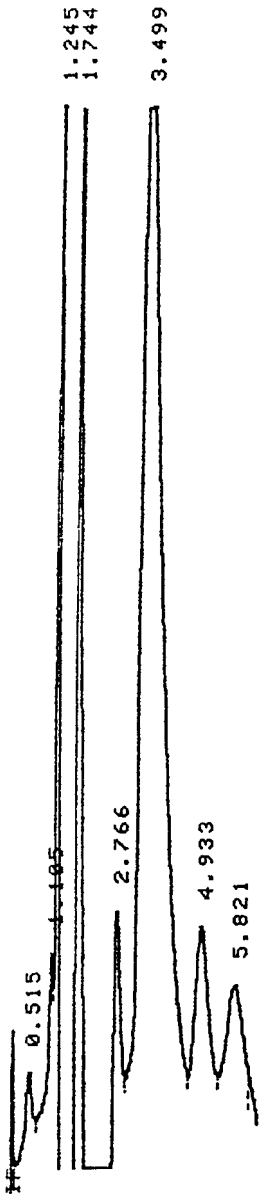

Figure 3. High performance liquid chromatogram of analysis of glycine (2.766 min), *N*,*N*-DMG (3.499 min), and glycine betaine (4.933 min) in spiked human blood serum samples with 7-methylxanthine (5.821 min) as internal standard. Chromatographic conditions are described in text.

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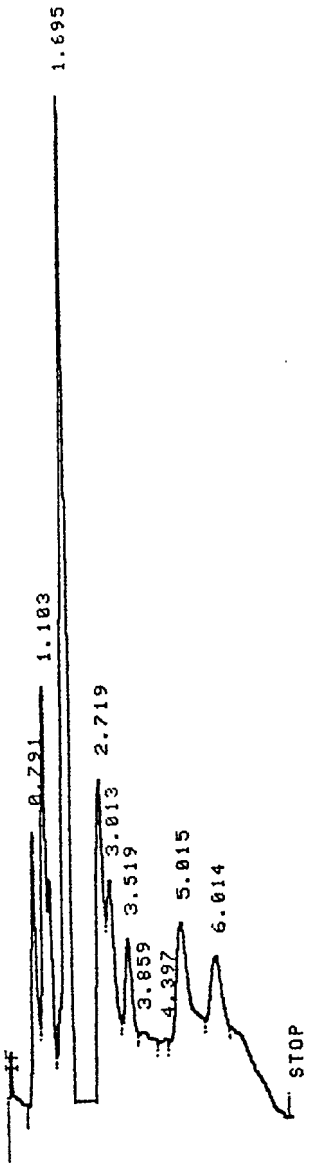

Figure 4. High performance liquid chromatogram of analysis of glycine (2.719 min), *N*,*N*-DMG (3.519 min), and glycine betaine (5.015 min) in urine samples with 7-methylxanthine (6.014 min) as internal standard. Chromatographic conditions are described in text.

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Table 5. Recovery of *N*,*N*-Dimethylglycine and Glycine Betaine from Human Blood Serum and Urine

	Serum				Urine			
Added (ng)	Found (ng)	SD (ng)	RSD (%)	Recovery (%)	Found (ng)	SD (ng)	RSD (%)	Recovery (%)
N,*N*-DMG								
50.00	46.70	4.32	9.2	93.4	46.78	4.27	9.1	93.6
100.00	106.57	6.08	5.7	106.6	116.22	7.88	7.4	106.2
250.00	227.25	6.16	2.7	90.9	243.64	20.77	8.5	97.5
Glycine betaine								
50.00	47.82	5.59	11.7	95.6	50.45	4.74	9.4	100.9
100.00	152.60	8.50	5.8	101.7	103.45	7.11	6.9	103.4
250.00	250.79	22.38	8.9	100.3	259.80	26.11	10.0	103.9

chose sample dilution and subsequent standard addition, which would be necessary in either case.

The precision and accuracy studies from biological samples were conducted by spiking blood serum and urine samples with three known concentrations of the compounds and then by comparing the obtained results with those as calculated from regression equations. Results of recovery studies for serum and for urine samples are given in Table 5. Each value represents the mean of six measurements carried out.

CONCLUSIONS

Because glycine betaine is often given orally to patients with metabolic disorders, knowing the levels of glycine betaine and its metabolite in urine and blood serum is a useful tool for studies on betaine metabolism and for individualization and optimization of therapy of homocystinuria. Rapid and reliable results are required for monitoring concentrations during therapy.

A simple, rapid, and direct method for the simultaneous determination of glycine betaine and its metabolite has been developed. This method has been tested on samples of blood serum and urine, as well as on pharmaceutical preparations. The method is useful in clinical assays, in routine pharmacological analysis, and in monitoring of biological fluid concentrations following the administration of an oral dose during treatment. The advantages of the proposed method over previously reported ones are basically the facts that no complicated derivatization steps are necessary and that it is simple and highly sensitive.

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