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Simultaneous determination of gluconolactone, galactonolactone and galactitol in urine by reversed-phase liquid chromatography: application to galactosemia

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ABSTRACT

A reversed-phase liquid chromatographic assay was developed for the specific evaluation of metabolic by-products in the urine of galactosemic patients and based on the simultaneous determination of gluconolactone, galactonolactone and galactitol. The procedure involved a lyophilization step and the formation of phenylisocyanate derivatives, followed by injection directly into the chromatograph. Analytical results showed good selectivity, linearity, precision and accuracy. The method enabled the detection of levels as low as 0.05–0.1 ng, and compared favourably with other published techniques for the estimation of aldonic acids in biological fluids.

INTRODUCTION

It has been suggested that an alternative route of galactose metabolism in galactosemic patients is oxidation to galactonolactone. This metabolic by-product has been found in the urine of patients and in the tissues of galactose-fed animals. Another pathway of galactose metabolism is the reduction to galactitol [1]. Different methods, including gas chromatography, have been described for the identification of galactonolactone in biological fluids in order to investigate this galactose oxidation pathway [2,3]. However, not all the systems differentiate between galactonolactone and its epimer gluconolactone. Consequently, additional separate studies have been performed to ensure the absence of interference from gluconolactone.

The aim of our study was to develop and validate a sensitive, selective high-performance liquid chromatographic (HPLC) method for the simultaneous routine determination of gluconolactone, galactonolactone and galactitol in urine. Reversed-phase HPLC was applied after derivatization with phenylisocyanate. The phenylurethanes formed were detected at 240 nm.

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EXPERIMENTAL

Reagents

Sugars were obtained from Sigma (La Verpillière, France). All mobile phases were prepared from HPLC-grade solvents (Carlo Erba, Paris, France). The reagents used for derivatization (dimethylformamide, phenylisocyanate and methanol) were provided by Prolabo (Paris, France).

Apparatus

Samples were lyophilized in a Serail lyophilizator (capacity 100 ml) at −50°C for 18 h. Derivatization was carried out in a thermostatted block (code 92617, Bioblock Scientific, Illkirch, France).

The liquid chromatograph used was a Spectra-Physics system (Les Ulis, France) equipped with a pump (Model 8700XR), a Rheodyne injector (10-μl loop) and an integrator (Model 4200). A Shimadzu SPD2A UV detector from Touzart et Matignon (Vitry sur Seine, France) was used.

Derivatization technique

A pool of urine samples were collected from male, female and neonates and spiked with known amounts of gluconolactone, galactonolactone and galactitol. Lyophilization of many aliquots (5 ml) was performed, and the dried samples were stored in a vacuum desiccator. Before the analysis, the residue was reconstituted with 5 ml of dimethylformamide. After centrifugation at 3000 *g* for 10 min, 1 ml of the supernatant was derivatized by adding 0.3 ml of phenylisocyanate and heating at 100°C for 1 h. Derivatization was stopped by adding 0.5 ml of methanol, and the mixture was appropriately diluted with dimethylformamide. The resulting solution was injected into the chromatograph. Urine from patients was treated in a similar manner. Quantitation was achieved by measuring the peak areas of the standard and the sample.

Chromatographic conditions

The separation was carried out on a Brownlee Labs. ODS 224 RP18 column (5 μm, 220 mm × 4.6 mm I.D.) from Touzart et Matignon. The isocratic eluent consisted of acetonitrile–water (60:40, v/v) at a flow-rate 2.0 ml/min. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

Methods design

Pretreatment. Lyophilization of urinary samples has been applied and is well adapted to the derivatization technique with phenylisocyanate: water, which reacts with this reagent, was removed. Moreover, many aliquots could be treated simultaneously and conserved for several months in a vacuum desiccator.

Derivatization. Phenylisocyanate reacts with hydroxyl groups of alcohols, phenols and sugars to yield phenylcarbamate esters (phenylurethanes) and has been used as a derivatizing agent prior to HPLC analysis [4,5]. The resulting stable and strongly UV-absorbing compounds were separated by the reversed-phase technique, but aldonolactones have not been investigated. In the present method, the reaction was carried out in dimethylformamide owing to its good catalytic properties. Various temperatures, reaction times and amounts of phenylisocyanate were tested, based on the results obtained after optimization. The optimal conditions were found to be at 100°C for 1 h with a volume ratio of reaction medium to phenyl isocyanate of 1:0.3. The corresponding derivatization gave rise to a major chromatographic peak from each sugar. Structural analysis of the phenylurethanes formed and the exact nature of the minor and major peaks from each aldonolactone will be discussed elsewhere [6].

Fig. 1 shows a comparison of a chromatogram obtained from blank urine and from a standard urine spiked with gluconolactone, galactonolactone and galactitol. Because it eluted constantly at a suitable position on the chromatogram (6 min), the peak R was chosen as the reference compound to calculate the relative retention time of each derivative. This peak corresponded to a side product, the *sym*-triphenylbiuret produced from the reaction between phenylisocyanate and traces of water.

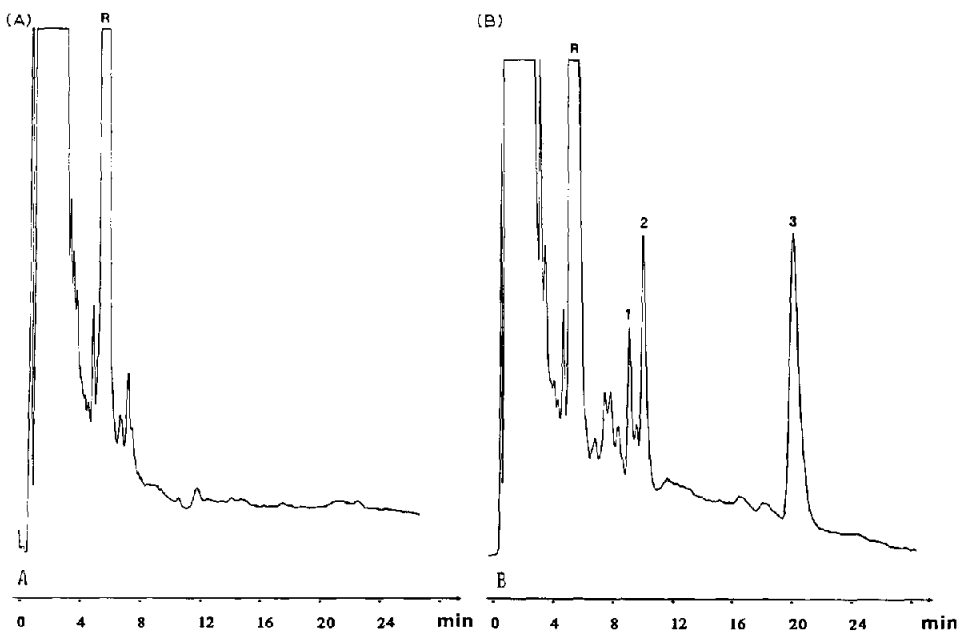

Fig. 1. Chromatograms of (A) blank urine sample and (B) standard urine sample spiked with the analysed sugars (0.5 mg/ml of each compound, 0.08 μg injected). Peaks: R = reference compound; 1 = gluconolactone; 2 = galactonolactone; 3 = galactitol.

The relative retention times of the major derivatives of the tested sugars were 1.82, 2.02 and 4.16, respectively. Quantitation was made with the major peak obtained from each sugar, and a good selectivity of the chromatographic separation was observed. The following method validation demonstrated that the minor peaks produced from aldonic acids did not affect the elution of the major peaks.

Method validation

Selectivity. Several blank urine samples from different subjects were tested for the absence of interfering endogenous components. Fig. 1A shows a typical chromatogram of a sugar-free urine: no interfering peaks at the retention times of the relevant components are observed.

In addition, possible chromatographic interferences from other sugars that could be present in the urine of galactosemic patients were checked. No interference was found when blank urine samples spiked with monosaccharides and other alditols were processed according to the described method. A corresponding chromatogram is shown in Fig. 2.

It may be mentioned that ketoses and disaccharides did not interfere with the analysis because they are not sensitive to the optimal conditions of derivatization reported.

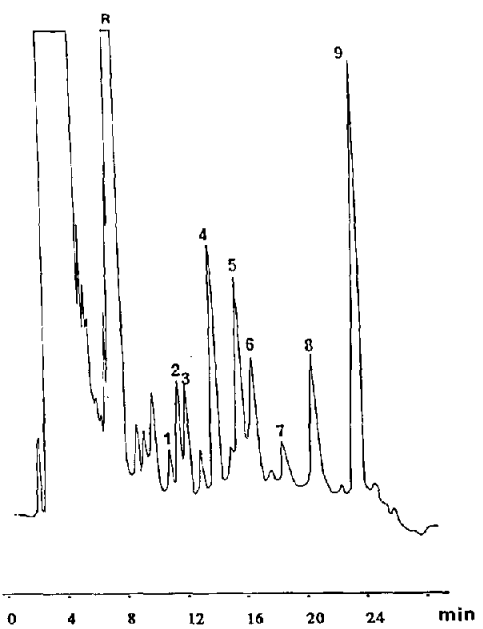

Fig. 2. Chromatogram of urine sample spiked with different sugars. Peaks: R = reference compound; 1 = gluconolactone; 2 = glucose; 3 = galactonolactone; 4 = glucose + galactose; 5 = allose; 6 = galactose; 7 = myoinositol; 8 = galactitol; 9 = sorbitol + mannitol.

Limit of detection. The detection limits for gluconolactone, galactonolactone and galactitol were 0.4, 0.2 and 0.05 ng, respectively, at a signal-to-noise ratio of 3:1.

Linearity. For calibration, the external standard method was chosen. The calibrators were prepared by the addition of gluconolactone, galactonolactone and galactitol in blank urine samples to give initial concentration levels between 0.1 and 0.6 mg/ml. The resulting samples were derivatized and analysed by the procedure described above.

The calibration curves were constructed by plotting the peak area *versus* the sugar concentrations. For both sugars, a linear relationship was obtained. The corresponding regression equations were $y = 349x + 1.66$ ($n = 12$, $r = 0.990$) for gluconolactone, $y = 477x + 1.70$ ($n = 12$, $r = 0.997$) for galactonolactone and $y = 1157x - 25.74$ ($n = 12$, $r = 0.999$) for galactitol.

Precision. The precision of the method was evaluated by making multiple injections of a urine sample spiked with 0.5 mg/ml each of gluconolactone, galactonolactone and galactitol and calculating the peak areas. The within-day precision of the standard sample corresponded to coefficients of variation of 2.92, 2.47 and 0.88%, respectively ($n = 6$). The between-day precision values were 3.49, 3.50 and 1.34%, respectively ($n = 9$).

Accuracy. The accuracy was evaluated from the calibration curves using weighed amounts of the three compounds added to urine (0.5 and 0.2 mg/ml of each sugar). The experimental mean was compared with the known value: Student's statistical test revealed that in all cases the null hypothesis was retained and there was no evidence of systematic error, the observed value of t (t_{exp}) was less than the critical value at the 0.05 level (degrees of freedom = 4, $t = 2.78$). The corresponding results are expressed in Table I.

TABLE I

ESTIMATION OF ACCURACY USING SPIKED URINE SAMPLES

Added (mg/ml)	Found (mean ± S.D., $n = 5$) (mg/ml)	C.V. (%)	t_{exp}
Gluconolactone			
0.5	0.473 ± 0.0238	5.04	2.27
0.2	0.187 ± 0.0102	5.45	2.549
Galactonolactone			
0.5	0.491 ± 0.0198	4.04	0.909
0.2	0.213 ± 0.0115	5.42	2.260
Galactitol			
0.5	0.499 ± 0.0102	2.06	0.196
0.2	0.193 ± 0.0063	3.29	2.222

Comparison with other methods

Galactonolactone and galactitol have been isolated in biological fluids and tissues of galactosemic patients by means of various chromatographic methods, such as thin-layer chromatography and gas chromatography (GC) [1,2,7,8].

GC using trimethylsilyl derivatives is commonly used for the identification and quantification of the by-products of galactose. This method provides adequate sensitivity but suffers from the lack of selectivity between galactonolactone and its epimer gluconolactone, which may be a potential interfering substance. Consequently, the authors [2] reported additional means of characterization to differentiate the epimers involving lengthy and cumbersome sample work-up (paper chromatography, decomposition point of purified benzimidazole derivatives).

Rogers *et al.* [7] proposed a specific identification by derivatization with butylboroacetic acid before GC analysis. Unfortunately, this technique is not sufficiently sensitive for quantifying the aldonic acids.

Recently, an excellent method of converting aldonic acids into N-propylamide acetate derivatives followed by GC was described by Lehrfeld [9], which permitted the separation of the two epimers. However, it involved a considerable number of pretreatment steps.

Ion-exchange HPLC has been investigated for the analysis of aldonic acids [10], but the method did not allow the separation of gluconic acid and galactonic acid.

Therefore, the present study overcomes these difficulties and offers the combined advantages of sensitivity, specificity and selectivity, together with the ability to perform multiple determinations using a single sample.

Application

A one-month-old male infant with dehydration and hepatic failure was admitted to the hospital. Immediately after diagnosis of galactosemia, the patient received the appropriate treatment with a diet restricted in galactose. A 24-h urine sample after twelve days of treatment was assayed by the present method. The corresponding chromatogram (Fig. 3) and quantification demonstrated that the patient excreted a significant amount of galactitol (19.2 mg per 24 h). This result is not surprising because continuing galactitol excretion after a restricted diet has been proved [1].

Urinary excretion of galactonolactone was not observed in our sample and was attributed to the twelve days of treatment.

These preliminary results serve only to focus attention for medical research. The simultaneous determination of the three sugars will depend on further random collection of urine samples from galactosemic patients before and after treatment. This contribution will be useful for exploration of the minor pathways of galactose metabolism and in clinical investigations and may be successfully used for routine analysis.

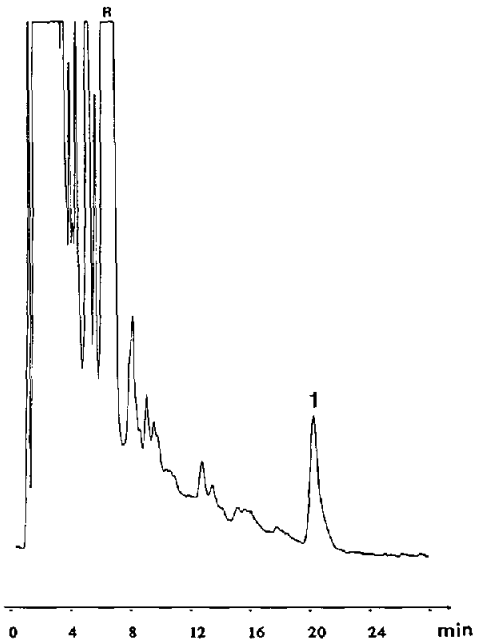

Fig. 3. Chromatogram of a urine sample from a treated patient. Peaks: R = reference compound; 1 = galactitol.

CONCLUSION

Gluconolactone, galactonolactone and galactitol were determined in urine by reversed-phase HPLC with good precision and accuracy. In comparison with other methods, this analysis is very simple, highly selective and sensitive. The reported separation should enable quantitative examination of the metabolic by-products in galactosemia. It also seems to be promising for clinical investigations and routine use.

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