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Short Communication

Benzoylation of sugars, polyols and amino acids in biological fluids for high-performance liquid chromatographic analysis

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Abstract

A precolumn benzoylation for analyzing sugars, polyols and neutral amino acids in biological fluids by high-performance liquid chromatography has been developed, which avoids protein precipitation, drying procedures and the use of pyridine. Derivatization and chromatography can be performed within one hour with a minimum detectable quantity of *ca.* 1 pmol (signal-to-noise ratio >2). The derivatization products of glucose, mannitol and neutral amino acids were identified by electrospray mass spectrometry (ES-MS) to be tetra- and pentabenzoyl glucose, penta- and hexabenzoyl mannitol and 2-phenyl-5-benzoyloxyoxazoles, respectively.

1. Introduction

The benzoylation procedure covers a broad spectrum of hydroxyl- and amino group containing substances, proceeds in the presence of water and gives UV-active derivatives suitable for extraction with organic solvents [1,2].

These features prompted us to investigate whether benzoylation can be used as a precolumn derivatization procedure for the simultaneous HPLC analysis of several amino acids, sugars and polyols in biological samples. The procedures available to date are not well suited for exploratory investigations, because of laborious sample preparation or recovery of only a limited group of substances.

Our investigations led to the development of a simple and rapid HPLC determination of sugars, polyols and amino acids based on reaction with benzoyl chloride directly in biological fluids, which is described in the present work.

2. Experimental

2.1. Materials

Benzoyl chloride, triethylamine (for biochemical purposes), ethyl acetate and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany) and used without further purification. Dulbecco's Modified Eagle Medium (DMEM) and foetal bovine serum were purchased from Biochrom (Berlin, Germany) and Sigma (Deisenhofen, Germany) respectively.

2.2. Mass spectrometry

Aqueous solutions (20 m*M*) of glucose, mannitol and the investigated amino acids were

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reacted as described under *Derivatization*. The separated HPLC fractions containing the benzoylation products were analyzed directly in the HPLC solvent by electrospray mass spectrometry. Mass spectra were obtained with a quadrupole mass spectrometer TSQ 700 (Finnigan MAT, Bremen, Germany) equipped with an electrospray ionisation source. The sample was introduced by syringe infusion into the electrospray ion source with a flow-rate of 1 μl/min. The ion source was operated without sheath liquid at a drying gas temperature of 140°C and a high voltage of 2.8 kV.

2.3. Samples for HPLC analysis

As samples were utilized foetal bovine serum, DMEM containing 10% foetal bovine serum and solutions of the analytes in DMEM containing 10% foetal bovine serum.

2.4. Chromatographic conditions

HPLC was performed using a Bischoff HPLC-gradient system (Leonberg, Germany) equipped with a Kromasil 100 C_{18}, 5-μm column (250 × 4 mm I.D.), precolumns containing the same sorbent and a Rheodyne RH 8125 injection valve with a 50-μl sample loop. Elution was carried out with acetonitrile–water mixtures (gradient from 70 to 95% acetonitrile within 30 min) at a flow-rate of 1.0 ml/min. Detection was performed at 228 nm (sugars and polyols) and 274 nm (amino acids) and the peaks were quantified using a Hyperdata Integration Workstation S I (Bischoff, Leonberg, Germany).

2.5. Derivatization

Sugars and polyols

To 70 μl of the neutral sample solution containing 0.2–2000 nmol of the hydroxyl compound and 10–20 μg serum protein 10 μl D-glucosamine-HCl solution (1 m*M*), 20 μl K_2HPO_4 solution (1 *M*), 10 μl benzoyl chloride and 20 μl (to favour the formation of incomplete benzoylated species) or 25 μl (to favour the formation of fully benzoylated derivatives) NaOH (8 *M*) were added. Immediately after the addition, the reaction solution was vortex-mixed for 5 min at 2500 vibrations/min using 1.5-ml polypropylene centrifugation tubes (Tecnomara, Fernwald, Germany). Subsequently the mixture was neutralized with *ca.* 10 μl H_3PO_4 (1.4 *M*) and after addition of 100 μl ethyl acetate vortex-mixed for an additional minute at 2500 vibrations/min. Finally 25 μl of the ethyl acetate phase were mixed with 100 μl acetonitrile–water (7:3, v/v) and the sample was immediately analysed by HPLC.

Alternatively, in order to enhance the sensitivity in the lower concentration range, 75 μl of the ethyl acetate phase were evaporated and the residue dissolved in 100 μl of acetonitrile–water (7:3, v/v) was then subjected to HPLC.

Amino acids

To 90 μl of the neutral sample solution containing 0.2–2000 nmol of the amino acids 10 μl cyclohexylalanine solution (2.5 m*M*) and 200 μl ethyl acetate followed by 10 μl benzoyl chloride and 30 μl triethylamine were added. Subsequently the mixture was vortex-mixed for 2 min at 2500 vibrations/min. After mixing of 25 μl of the ethyl acetate phase with 100 μl acetonitrile–water (7:3, v/v) HPLC analysis was performed immediately.

With samples in the lower concentration range containing up to *ca.* 50 nmol of the amino acid only 2 μl benzoyl chloride and 6 μl triethyl amine were used for the reaction and 150 μl of the resulting ethyl acetate phase were evaporated. Subsequently the residue was dissolved in 100 μl acetonitrile–water (7:3, v/v) and analysed by HPLC.

3. Results

Reaction with *ca.* 100-fold excess of benzoylchloride/NaOH for a few min directly in cell culture media resulted in a reproducible benzoylation of sugars and polyols. The reaction products proved to be stable for several hours, both in ethyl acetate and in acetonitrile–water (7:3, v/v), thus enabling quantitation by HPLC.

The HPLC-peaks of the reaction products of D-glucose and mannitol, investigated as repre-

Table 1
HPLC and MS characteristics of the benzoylation products of several sugars and polyols

Substance	t_R (min)	R^2 [a]	ES-MS[b] (m/z)
Benzyl alcohol	6.1	0.990	
D-Glucose	7.5[c]/7.9[c]/ 8.1[c]		619/635 $[M+Na/K]^+$ [c]
	15.6[d]/15.9[d]		723/739 $[M+Na/K]^+$ [d]
D-Glucosamine	8.8/9.7		
Adenosine	10.9		
Mannitol	11.3[d]/11.5[d]	0.945[d]	725/741 $[M+Na/K]^+$ [d]
	18.7[e]	0.980[e]	829/845 $[M+Na/K]^+$ [e]
2-Desoxy-D-glucose	11.6/12.1	0.990	
3-O-Methyl-glucose	12.1		
cytidine	12.3		
Myoinositol	17.7		
Sucrose	14.0/14.7/ 19.5/20.0/ 20.8/26.2		

[a] R = regression coefficients of the calibration lines composed of 12 determinations at different concentrations. To obtain the calibration line these substances were dissolved together in the cell culture medium DMEM containing 10% fetal bovine serum to a concentration of 10 mM each (mannitol and benzyl alcohol each 20 mM). Samples (10 μl) of this stock solution and from dilutions with DMEM/10% serum were analyzed as described under Experimental.
[b] Nominal masses.
[c] Tetrabenzoyl.
[d] Pentabenzoyl.
[e] Hexabenzoyl derivative.

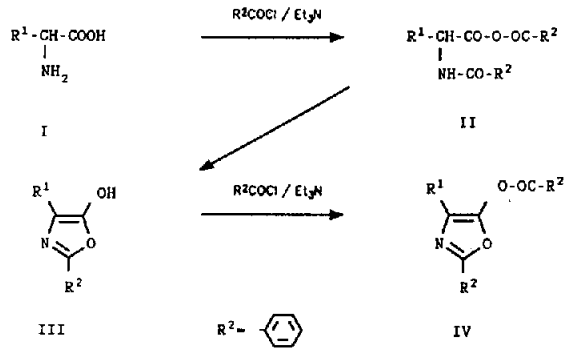

Fig. 1. Reaction scheme showing the formation of derivatives III and IV.

sentatives for hydroxyl containing compounds, correspond to tetra- and pentabenzoyl glucose and penta- and hexabenzoyl mannitol, according to ES-MS (Table 1). The tetra- and pentabenzoyl glucose as well as the penta- and hexabenzoyl mannitol could be used independently and with comparable results for the quantitation of glucose or mannitol, respectively.

The ratio of both reaction products of the given substance could be influenced by the amounts of NaOH and serum proteins contained in the reaction mixture. Excess NaOH favoured the formation of the fully benzoylated products, whereas the presence of serum proteins impaired this reaction. The effect of serum proteins could already be observed at a protein concentration of *ca.* 1 μg/ml and reaches its maximum level at *ca.* 50 μg/ml with no further increase up to 500 μg/ml. This result suggests a detergent effect rather than a competition of the proteins with the substrates for the reagent.

If the protein concentration was maintained in the range of 100–200 μg/ml by addition of serum, the effect of the serum proteins was nearly constant and reliable results could be achieved also with samples containing different amounts of serum proteins. Addition of human serum albumin showed principally the same effects.

The benzoylation products of the amino acids could be extracted from the reaction mixture with ethyl acetate after acidification. However, in this case the HPLC analysis (acetonitrile–0.1% TFA gradients) was disturbed by the benzoylation products of serum proteins which were also extracted from the acidified reaction mixture.

Modification of the reaction conditions, to overcome this problem, resulted in a kind of phase-transfer reaction using a mixture of the sample with ethyl acetate and triethylamine (Et_3N) instead of NaOH (see Experimental). For amino acids, other less polar derivatization products were found after this procedure in the ethyl acetate phase than those obtained by the first approach. Benzoylation products of hydroxyl-containing substances and of serum proteins could not be detected in the ethyl acetate phase after this procedure.

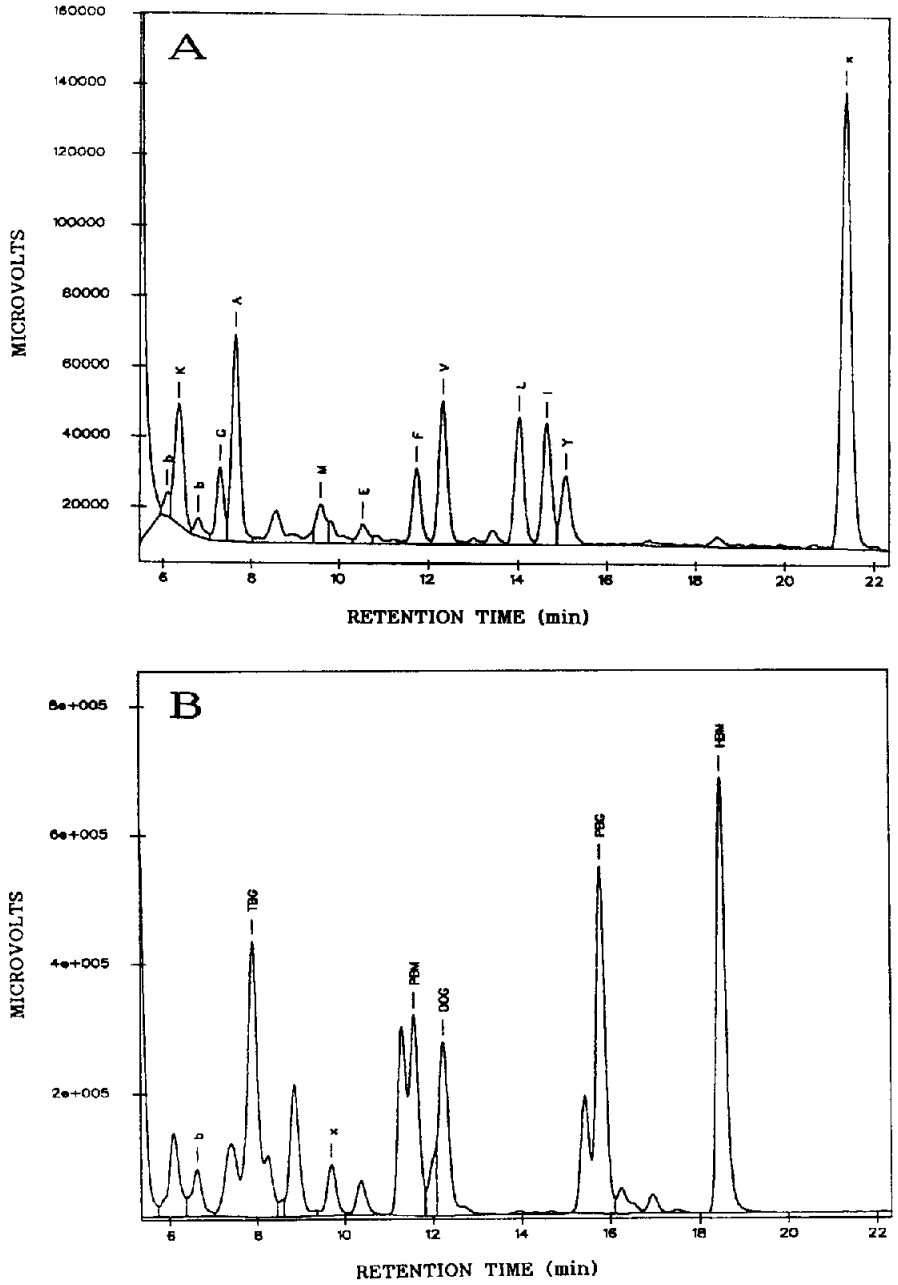

Fig. 2. HPLC analysis of DMEM containing 10% fetal bovine serum supplemented with alanine (1 m*M*), 3-O-methylglucose (5 m*M*) and mannitol (5 m*M*). Ten microliters were modified according to the procedure described in Experimental after addition of 80 μl (amino acids) or 60 μl (polyols) of water. Peaks: x = internal standards (cyclohexylalanine or glucosamine, respectively), b = by-products originating from excess reagent. (A) Amino acid derivatives (one letter abbreviations). (B) Derivatives of sugars and polyols; TBG = tetrabenzoyl-D-glucose, PBG = pentabenzoyl-D-glucose, PBM = pentabenzoyl mannitol, HBM = hexabenzoyl mannitol, DOG = 3-O-methyl-D-glucose.

The products derived from neutral amino acids were stable in ethyl acetate and in acetonitrile–water mixtures for at least several hours and could be separated easily under the same HPLC conditions as those used for the sugar- and polyolderivatives.

The ES-MS spectra, recorded from separated HPLC fractions of several amino acids (Gly, Ala, Phe, Leu, Lys) modified in this way, showed intensive signals corresponding to the $[M+H]^-$, $[M+Na]^+$ and $[M+K]^+$ peaks of 4-substituted 2-phenyl-5-benzoyloxyoxazol derivatives (IV, Fig. 1, Table 2). Identification of this structure is supported further by the facts, that the reaction conditions used generally favour the formation of oxazolinones-(5) [3] and the subsequent acylation of their tautomers [4], and that peaks corresponding to simple N-benzoylated amino acids, the anhydrides II or the oxazolinones-(5) (III, Fig. 1), could not be observed in the MS spectra.

Some amino acids containing additional functional groups (Asp, Arg, Ser, Thr) could not be determined by the present method, because of the instability of their reaction products in the elution mixture and their short retention times leading to interference with side products from the benzoylation reaction (benzoic anhydride and several non-identified compounds appearing in the HPLC chromatograms in the range of 2–6 min). Despite its instability in the elution mixture quantitation of the benzoylation product of glutamic acid was possible with a reliability comparable to that of the neutral amino acids when the time needed to dissolve the sample in the eluent and to inject the sample onto the column was kept constant.

From the UV intensity of the HPLC peaks an approximately quantitative reaction of the sugars, polyols and amino acids listed in Tables 1 and 2 can be concluded, taking into account an extinction coefficient at 228 nm of *ca.* 10 000 as can be found for simple benzoic acid amides and esters. Reversed-phase HPLC (see Experimental) resulted in sharp peaks both for the oxazol derivatives (Fig. 2A) and the full benzoylated polyols, and in groups of peaks for the sugars and not quantitatively benzoylated polyols (anomers and isomers, respectively; Fig. 2B) with a detection limit in the range of 1–5 pmol

Table 2
HPLC and MS characteristics of the benzoylation products of several amino acids

Substance	t_R[c] (min)	R^{2a}	ES-MS[b] (m/z)	
Lysine	6.4	0.976	441/463	$[M+H/Na]^+$
Glycine	7.1	0.984	266	$[M+H]^+$
Alanine	7.5 ± 0.3	0.986	280	$[M+H]^+$
Glutamate	10.4	0.997		
Tryptophane	8.5			
Cystine	8.5			
Methionine	9.4			
Phenylalanine	11.8	0.976	356/378	$[M+H/Na]^+$
Valine	12.1			
Cysteine	13.3			
Leucine	13.9	0.980	322/344	$[M+H/Na]^+$
Isoleucine	15.1 ± 0.5			
Naphthyl-alanine	15.2			
Tyrosine	15.5			
Cyclohexyl-alanine	21.0 ± 0.5			

[a,b] See footnotes of Table 1.
[c] Retention times given with S.D. represent the mean of 12 determinations.

(signal-to-noise ratio >2). Linear calibration plots (peak area at 274 nm or 228 nm, respectively, *vs.* amount of compound) were obtained in a range corresponding to 0.01–10 nmol modified analyte injected onto the column both for samples of the stock solutions and for dilutions

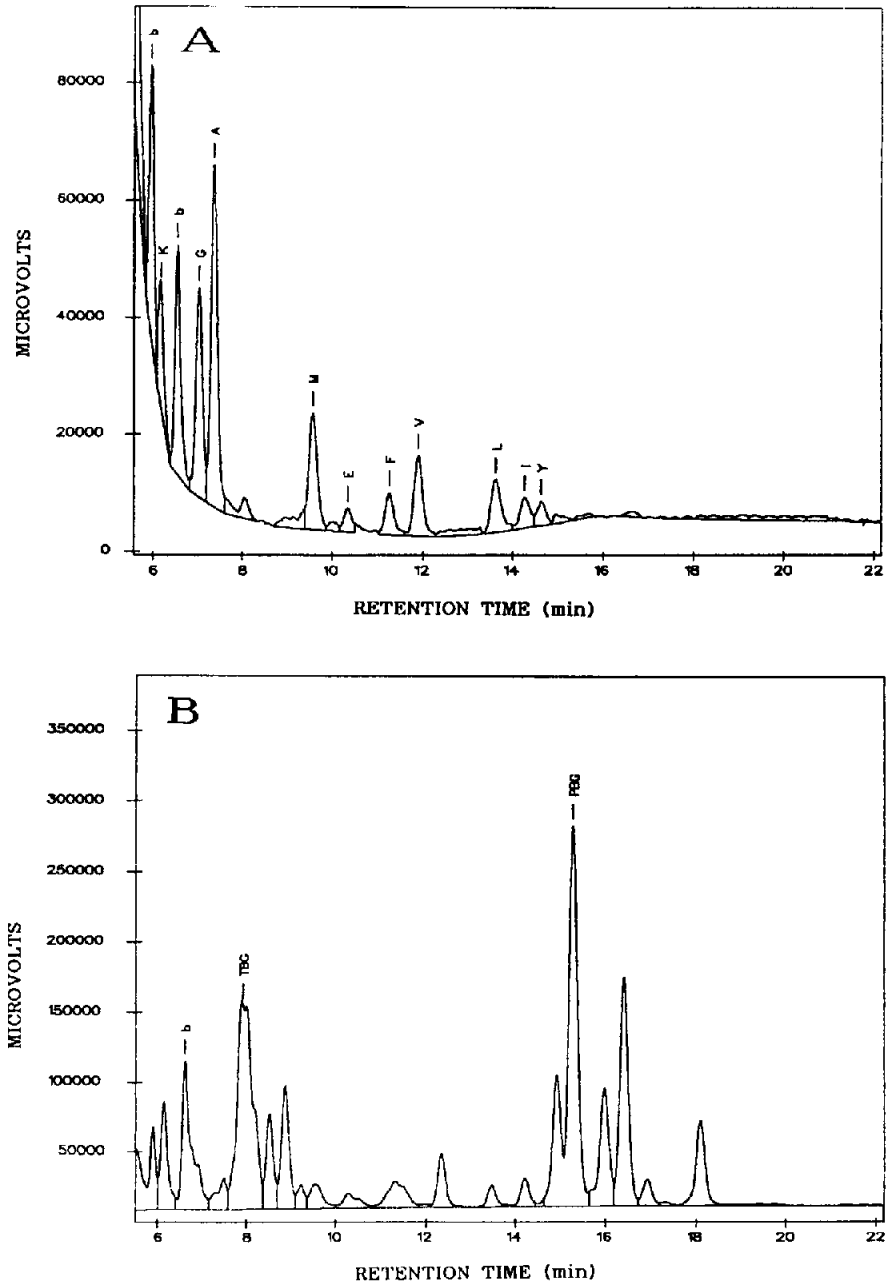

Fig. 3. HPLC analysis of fetal bovine serum. One microliter was modified according to the procedure described in Experimental using the low-concentration variant after addition of 89 μl (amino acids) or 69 μl (polyols) of water. (A) Amino acid derivatives. (B) Derivatives of sugars and polyols. For peak identification see Fig. 1.

with DMEM–10% serum (Tables 1 and 2). Sensitivity and limitations of the present method are illustrated in Fig. 3 displaying several peaks in the range of the quantitation limit of *ca.* 10 pmol (*e.g.* that of isoleucine corresponds to an amount of 8 pmol). Likewise this figure illustrates the utility of this approach for the analysis of small volumes of biological fluids.

4. Discussion

The usual benzoylation- and naphthoylation procedures for HPLC analysis of sugars and polyols need laborious sample preparations and reaction times of several hours [2,5]. In contrast, the benzoylation approach presented here requires only *ca.* 10 min, proceeds directly in the biological fluid and avoids protein precipitation or drying. As an additional advantage, the use of pyridine, which hampers the recovery in conventional procedures, is avoided in the present method.

In spite of the only moderate reliability and sensitivity (S.D.s in the range of 5–10% and *ca.* 10 pmol as quantitation limit), the substantially reduced efforts and the potential of covering amino acids as well as hydroxyl containing compounds render the present approach more appropriate for conducting rapid exploratory investigations than the specialized methods commonly used at present.

Moreover, the easy transformation of amino acids into 2-phenyl-5-benzoyloxyoxazoles, which are extractable by unpolar solvents, represents a general applicable complementation to the usual derivatization approaches for amino acids [6].

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6. References

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