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Simple and rapid method for determination of short-chain fatty acids in biological materials by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A new and versatile method for the identification and quantification of short-chain fatty acids, such as formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric and mercaptoacetic acids, in biological specimens by high-performance liquid chromatography is described. After sample purification by vacuum transfer and concentration by alkaline freeze-drying, the acids were measured without any further preparative step, using a sulphonated polystyrene–divinylbenzene column as stationary phase. Ultraviolet detection of the native acids was done at 214 nm. Peak resolution and reproducibility were as good as with gas chromatography. Many examples of the application of this method to a variety of biological specimens and fluids both from the rats and humans are described.

INTRODUCTION

Short-chain fatty acids (SCFAs) are the main end-products of anaerobic bacterial fermentation of carbohydrates in the human colon. The most important SCFAs in the human colon are acetic, propionic and butyric acids. Although the physiological importance of SCFAs in humans is not yet fully understood, there is evidence that they are of prime importance for the colonic mucosa in a number of ways: SCFAs represent a significant physiological energy source for the colonic mucosal epithelial cells [1], their lack has been claimed to be involved in the pathogenesis of ulcerative colitis [2] and diversion colitis [3], they may have regulatory functions in hepatic carbohydrate metabolism [4,5], and they might have some antineoplastic properties for the colon [6]. To study these effects in further detail, a valid and versatile method for the routine analysis of these metabolic products in biological specimens, such as intestinal tissues, serum, saliva and faeces, is considered a prerequisite. Furthermore, the rapid analysis of SCFAs may prove valuable for the diagnosis of small-bowel bacterial overgrowth in clinical gastroenterology [7,8].

Separation, identification and quantification of SCFAs have most commonly been done using gas chromatography (GC), either alone or in combination with mass spectrometry [9,10], after a sample pretreatment step (extraction with organic solutions or distillation techniques). These methods [11,12] are sensitive and specific, but time-consuming and expensive.

Based on different modes of separation and detection, high-performance liquid chromatography (HPLC) has been also applied to the analysis of carboxylic acids [13]. Therefore, the derivatization of SCFAs to UV-absorbing or fluorescing derivatives for monitoring SCFAs in biological matrices has been essential [14].

The aim of the present study was to establish a

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new HPLC method for the determination of SCFAs by using simple UV detection combined with sample purification by acid vacuum transfer, and to test the applicability of this analytical procedure to different relevant biological materials.

EXPERIMENTAL

Chemicals

Formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric and mercaptoacetic acids were obtained from Aldrich (Steinheim, Germany). The other chemicals and solvents were purchased from Merck (Darmstadt, Germany). All substances used were of the highest purity (HPLC grade). Water was purified with a Milipore Q system (Waters, Eschborn, Germany).

Chromatographic system

The instrumentation consisted of a Merck-Hitachi L-6000 pump, a Beckmann Promis II autosampler, a Beckmann D-160 UV detector and a Shimadzu CR 1B integrator. The column was placed in a Knauer constant-temperature column oven.

All separations of the SCFAs were performed isocratically on sulphonated polystyrene–divinylbenzene columns in hydrogen form (particle size 8 μm), with column dimensions of 300 mm × 9.5 mm I.D. (Polyspher OA HY or Polyspher KA HY; Merck). The mobile phase was 0.2 *M* sulphuric acid (pH 3.1). Separation of SCFAs was best with a flow-rate of 0.8 ml/min and a column temperature of 60°C. A single run required 35 min. Column regeneration was done after 500 runs by washing the column at 65°C with 25 m*M* ultrapure sulphuric acid at a flow-rate of 0.5 ml/min for 6 h. The native SCFAs were monitored at 214 nm.

Calibration curves were calculated on the basis of peak area using least-squares regression analysis.

Extraction of SCFAs from biological materials

The samples were pretreated by a slight modification of the method described by Pomare *et al.* [13]. Vacuum transfer was accomplished using a round-bottomed flask (5 cm external diameter), connected to a 10-ml conical tube (12 cm × 1.5 cm), and U-shaped connecting piece fitted with a glass tap. The glass joints and taps were lubricated with a hydrocarbon-based grease.

Duodenal secretions, saliva, plasma. Samples of fasting duodenal fluids were collected from patients during routinely performed gastroduodenoscopy. To 1 ml of the duodenal secretions or saliva, and plasma, 1 ml of 0.36 *M* perchloric acid and 1 μmol of 2,2-dimethylbutyric or isobutyric acid were added for deproteinization and as the internal standard, respectively. Together with 400 μl of 1 *M* potassium hydroxide solution, the acidified mixture was shell frozen in the conical tube using liquid nitrogen.

Tissue. Biopsies and tissue samples of human and rat colon were analysed. The tissue sample was diluted 1:10 in 0.9% sodium chloride, homogenized on ice with an ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at 19 000 rpm for four 14-s periods. To 1 ml of the homogenate, 1 μmol of internal standard was added. This solution was acidified and further prepared as described.

Faeces. The faecal samples and intestinal tissue were homogenized. A 0.5-g aliquot was weighed into a 12-ml plastic tube, with the addition of 10 ml of bovine serum albumin (BSA) solution (5 g per 100 ml) in order to increase the transfer efficiency [8]. After mixing on a vortex mixer (1 min) and centrifugation (1900 *g*, 10 min), 1 ml of the supernatant was pipetted into the round-bottomed flask, acidified and frozen at −196°C.

Vacuum transfer. Immediately after freezing, the flask and conical tube were connected to the U-tube and then evacuated to 0.1 mbar with a two-stage vacuum pump (Vacubrand, Wertheim, Germany). After evacuation (within 120 s), the conical tube was immersed in liquid nitrogen in a Dewar vessel (25 cm I.D., Isotherm-Werke, Karlsruhe, Germany), so that the lower 2 cm of the tube were covered. The flask was kept at room temperature, so that a temperature gradient for the transfer of volatile material from flask to tube was created. After 2 h, the vacuum

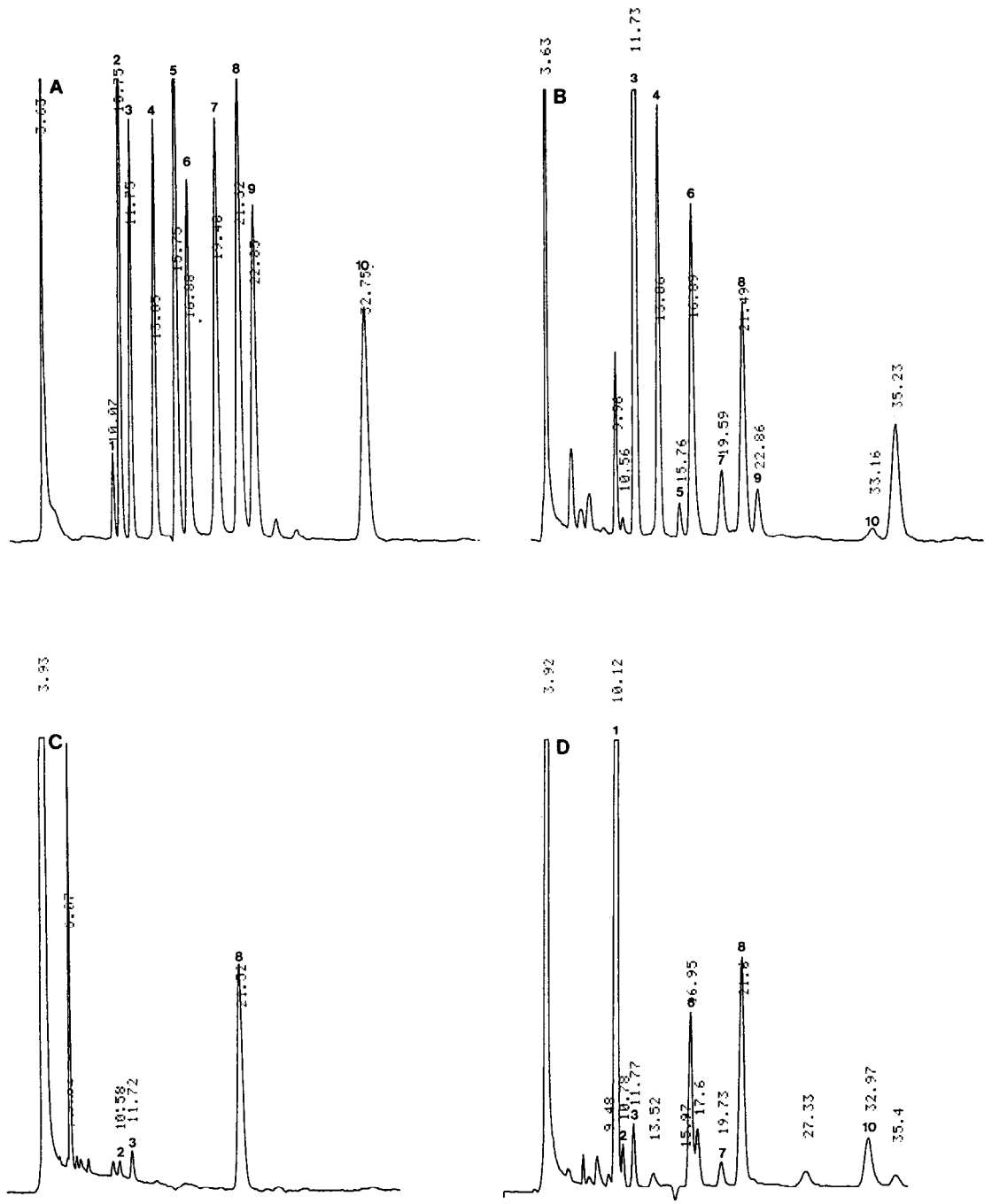

Fig. 1. Chromatograms of (A) standard SCFAs, with 2,2-dimethylbutyric acid as internal standard (50 nmol of each compound were injected), (B) human faeces, (C) duodenal aspirate from a healthy subject, (D) duodenal aspirate form a patient with small-bowel bacterial overgrowth (SBBO) due to chronic idiopathic intestinal pseudo-obstruction. Peaks: 1 = lactate; 2 = formic acid; 3 = acetic acid; 4 = propionic acid; 5 = isobutyric acid; 6 = *n*-butyric acid; 7 = isovaleric acid; 8 = 2,2-dimethylbutyric acid; 9 = *n*-valeric acid; 10 = caproic acid. Mobile phase, 0.2 *M* sulphuric acid (pH 3.1) at a flow-rate of 0.8 ml/min; injection volume, 50 μl; column temperature, 60°C; UV detection, 214 nm; column, Polyspher KA HY (8 μm) (300 mm × 9.5 mm I.D.).

was released, and the contents of the inner conical tube were thawed and mixed immediately, thus trapping the SCFAs in the alkaline solution. A 1-ml volume of this SCFA solution was transferred to micro-vessels, which were then placed in a Beckman autosampler. SCFAs standards were prepared from a stock solution (10 m*M* of each acid), and 0, 5, 10, 20, 50, 75 and 100 μl of the solution were added to 1 ml of BSA solution (5 g per 100 ml) to prepare a standard curve.

RESULTS AND DISCUSSION

Chromatography and detection

A typical chromatogram showing the separation of ten SCFAs with 2,2-dimethylbutyric acid as the internal standard is shown in Fig. 1. It is clear that the Polyspher OA HY column is able to separate all common SCFAs in less than 35 min. A similiar peak resolution is possible with the Polyspher KA HY column (data not shown).

The standard curves of SCFAs show a good linear correlation between peak-area ratios and the corresponding standard SCFAs over the range from 5 to 50 nmol, with *r* values of 0.993. The reproducibility (coefficient of variation, C.V.) of duplicate injections was found to be within 3%, when the peak-area ratios of the SCFAs to the internal standard were calculated. The lower limits of detection were in the region of 20 nmol/ml, when a 50-μl injection was made (signal-to-noise ratio 5:1). These detection limits are similar to those observed by other authors for GC [9,11] or HPLC coupled with a conductivity detector [13].

Reproducibility. The reproducibility was tested by dividing the supernatant of a random stool sample into ten identical aliquots, which were treated separately. The resulting C.V. ranged from 2.5 to 3.3%, when the peak-area ratio of the SCFAs to the internal standard were calculated (Table I).

Internal standard. We prefer to use 2,2-dimethylbutyric acid rather than isobutyric acid, which has been formerly used as internal standard [7,10,14], because in several studies [8,15,16] and also in this series (see below) isobutyric acid was shown to represent 5–10% of total normal faecal SCFAs.

Extraction procedure

As can be seen from Figs. 1 and 2, practically no impurities or interfering substances were found in the chromatograms of different biological matrices, thus indicating that vacuum transfer extraction is highly specific. This could also be confirmed by recording UV spectra of the different peaks, which were compared with those of

TABLE I

QUANTITATIVE CHROMATOGRAPHIC PARAMETERS FOR HPLC–UV ANALYSIS OF SCFAs

Compound	Retention time (min)	Range of linearity (nmol)	Linearity (*r* value)	Detection limit (nmol)	Analytical recovery, (mean ± S.D., *n* = 5) (%)	Reproducibility (μmol/g dry weight) (C.V., %)[a]
Formate	10.75 ± 0.23	10–500	0.994	2	102 ± 3.1	2.8 ± 0.09 (3.2)
Acetate	11.75 ± 0.33	20–500	0.995	2	98 ± 2.1	288.5 ± 8.65 (3.0)
Propionate	13.85 ± 0.21	20–500	0.996	2	96 ± 3.6	141.8 ± 5.65 (3.3)
Isobutyrate	15.73 ± 0.23	10–500	0.993	5	89 ± 1.8	58.5 ± 1.64 (2.8)
n-Butyrate	16.88 ± 0.27	20–500	0.994	5	101 ± 4.1	78.1 ± 2.0 (2.5)
Isovalerate	19.46 ± 0.30	20–500	0.996	10	92 ± 4.3	127.5 ± 5.65 (2.9)
2,2-Dimethylbutyrate	21.46 ± 0.22	30–500	0.997	10	98 ± 3.1	–
n-Valerate	22.83 ± 0.23	40–500	0.994	20	104 ± 5.1	6.3 ± 0.86 (4.3)
n-Caproate	32.73 ± 0.31	40–500	0.993	20	88 ± 4.1	N.D.

[a] Reproducibility is the percentage deviation of the mean as it has been obtained by repeated (ten) analyses of the same stool sample.

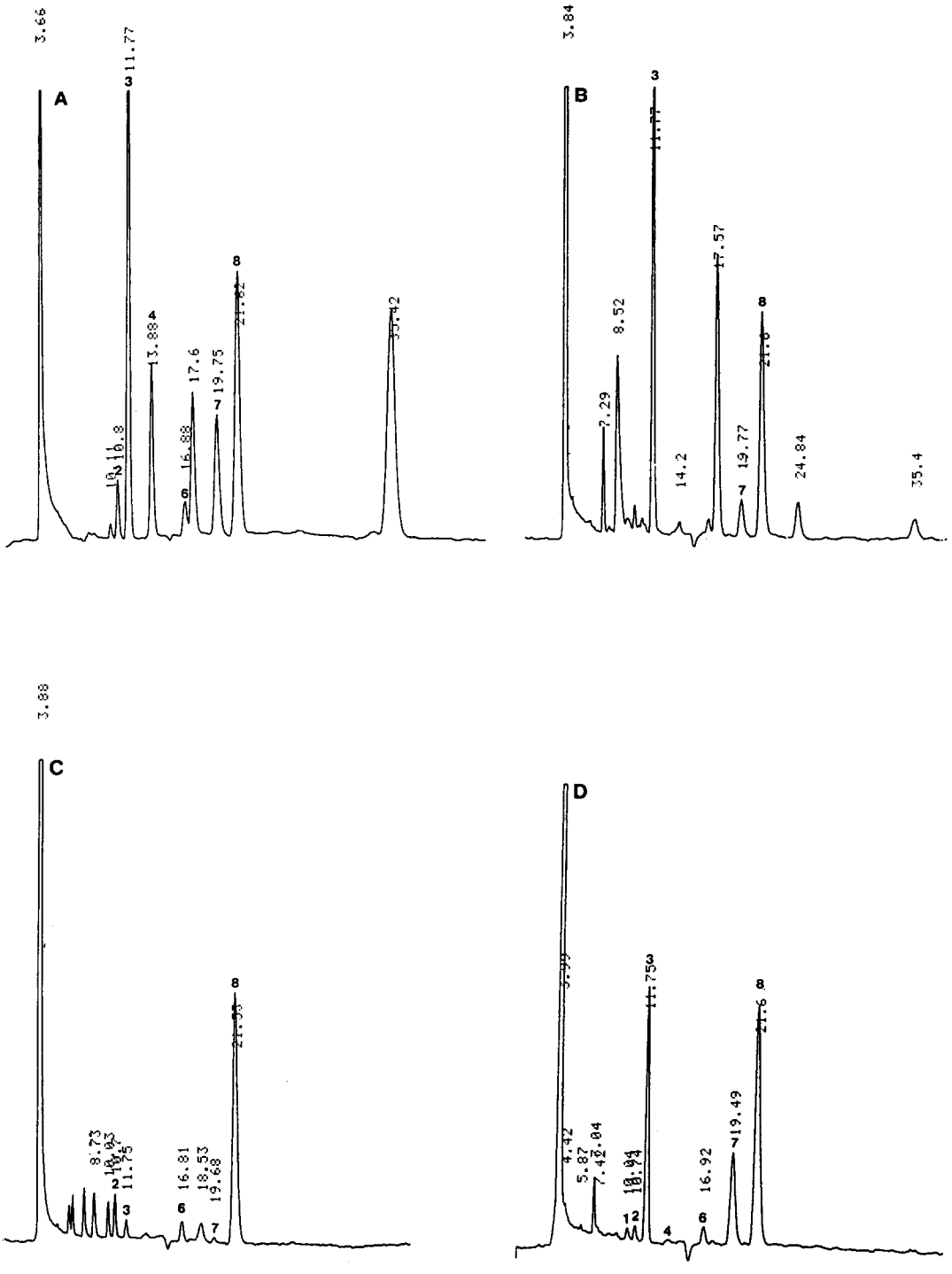

Fig. 2. Chromatograms of SCFAs after vacuum transfer extraction of biological specimens: (A) human saliva, 1 ml; (B) human urine 1 ml; (C) human plasma, 1 ml; (D) human colon, 100 mg. Each sample contained 1 μmol of 2,2-dimethylbutyric acid as internal standard. Peaks: 1 = unidentified; 2 = formic acid; 3 = acetic acid; 4 = propionic acid; 6 = *n*-butyric acid; 7 = isovaleric acid; 8 = 2,2-dimethylbutyric acid. Conditions as in Fig. 1.

TABLE II

FAECAL SCFAs IN HEALTHY SUBJECTS AND PATIENTS WITH CROHN'S OR CELIAC DISEASE

Compound	Control (n = 10)		Crohn's disease (n = 8)		Celiac disease (n = 4)	
	Amount (μmol/g dry weight)	Proportion (%)	Amount (μmol/g dry weight)	Proportion (%)	Amount (μmol/g dry weight)	Proportion (%)
Formate	1.83 ± 0.73	0.5	5.3 ± 0.94	2.1	3.9 ± 0.68	0.7
Acetate	161.9 ± 8.58	45.1	135.9 ± 6.58	54.7	268.4 ± 20.1	48.8
Propionate	90.9 ± 10.7	26.3	44.8 ± 2.78	18.0	149.0 ± 18.8	27.1
Isobutyrate	7.3 ± 1.84	2.0	11.8 ± 8.58	4.7	16.9 ± 1.08	3.1
n-Butyrate	73.9 ± 3.08	20.6	37.6 ± 2.58	15.2	84.1 ± 10.8	15.3
Isovalerate	12.7 ± 2.28	3.5	11.1 ± 1.92	4.5	10.7 ± 1.08	1.9
n-Valerate	6.92 ± 1.98	1.9	1.3 ± 8.58	0.5	12.6 ± 2.08	2.3
n-Caproate	3.94 ± 1.25	0.9	0.7 ± 0.58	0.23	4.9 ± 1.10	0.86
Total	359.4 ± 28.8	100	248.5 ± 8.38	100	550.5 ± 38.8	100

standards and found to be identical. Compared with other extraction methods that are in use, for sample preparation in GC analysis (ether extraction, steam distillation, cation-exchange extraction or ultrafiltration), this method has the advantage of being a simple, rapid and reliable pretreatment of samples for HPLC–UV.

Vacuum transfer of SCFAs avoids steam distillation, which can lead to decomposition of acetyl groups from other biological components and thus may account for falsely high acetate values [17].

Currently ether extraction is the routine method for the extraction of SCFAs. Nevertheless, appreciable amounts of acetic and propionic acids remain in the aqueous phase after extraction, resulting in relatively poor recoveries of these acids [11]. However the main disadvantage of this method, and also of ultrafiltration pretreatment, is interference with other compounds, which renders them inappropriate for HPLC–UV (data not shown).

Furthermore, vacuum transfer of SCFA has the advantage of concentrating the sample, which is especially necessary for human biopsies. Methodological problems that arose from the storage of biological SCFA samples [18] were avoided by using vacuum transfer immediately after the biological sample was obtained.

Recovery. To assess potential losses of SCFAs during the pretreatment procedure, 50 μl of a standard solution (200 nmol of each SCFA) were added to faecal samples and subjected to vacuum transfer. The absolute peak areas before and after were compared. The analytical recovery by the pretreatment procedure ranged from 88 to 104% (Table I).

Determination of SCFAs in different biological specimens

Determination of faecal SCFAs in healthy individuals and patients with Crohn's disease and coeliac disease. Stool samples were obtained from ten volunteers on regular diets. The mean concentration of total SCFAs in human faeces was 359.4 μmol/g dry weight (range 150–500 μmol/g dry weight) (Table II). Formic acid accounted for an average of 0.49%, acetic acid for 45.1%, propionic acid for 26.3%, butyric acid for 20.6% and isobutyric acid for 2.0%, respectively, of the total concentration. Isovaleric acid and valeric acid were also present in low concentrations. A typical chromatogram obtained from a specimen from a healthy volunteer is shown in Fig. 1.

The significantly reduced faecal SCFA content of patients with Crohn's disease (248.5 μmol/g dry weight) compared with the control group may arise from lower fibre intake, pharmacolog-

TABLE III

SCFAs IN HUMAN SALIVA, PLASMA AND DUODENAL SECRETIONS IN HEALTHY SUBJECTS AND IN A PATIENT WITH SMALL BOWEL BACTERIAL OVERGROWTH

Compound	Duodenal secretion				Saliva (n = 4)		Plasma (n = 6)	
	Healthy subjects (n = 4)		Bacterial overgrowth (n = 1)		Amount (μM)	Proportion (%)	Amount (μM)	Proportion (%)
	Amount (μM)	Proportion (%)	Amount (μM)	Proportion (%)				
Formate	43.0 ± 7.2	17.7	105.3	9.8	125.1 ± 14.1	8.7	48.8 ± 3.4	36.1
Acetate	69.9 ± 6.58	28.7	151.9	14.2	195.4 ± 16.1	13.4	57.8 ± 13.4	42.6
Propionate	32.1 ± 3.7	13.2	58.2	5.4	465.9 ± 38.8	31.9	N.D.	
Isobutyrate	10.0 ± 1.84	4.1	51.2	4.8	69.9 ± 8.08	4.8	N.D.	
n-Butyrate	33.3 ± 3.08	13.6	642.1	60.0	171.1 ± 23.8	11.6	28.7 ± 4.4	21.2
Isovalerate	55.1 ± 62.28	22.6	62.1	5.7	429.7 ± 54.8	29.6	N.D.	
Total	243.8 ± 28.8	100	1070.8	100	1456.2 ± 138.8	100	135.3 ± 24.6	100

ical treatment (antibiotics) or a critical lowering of the intraluminal pH, which shifts the bacterial metabolism from SCFAs to lactate production [19].

In contrast, patients with coeliac disease show a significantly higher faecal SCFA content (550.5 μmol/g dry weight), which may be the result of a higher colonic supply, with carbohydrates and fat as a principle consequence of maldigestion and impaired pancreatic secretion.

Determination of SCFAs in duodenal aspirate in case of small bowel bacterial overgrowth (SBBO) and in healthy subjects. Fig. 1 shows a typical chromatogram of SCFAs in duodenal secretions from patients without (C) and with (D) bacterial overgrowth. The mean concentration of total SCFAs in the patient with bacterial overgrowth was about four times higher (1070.8 *versus* 243.4 μM) than in duodenal aspirates from patients without overgrowth (Table III). This values are in agreement with those found by Hoeverstad *et al.* [8] using GC with flame ionization detection (FID). The relative distribution of the respective SCFAs was also very different, with relatively less acetic acid and more propionic, butyric and valeric acids in the patient with SBBO.

Determination of SCFAs in human saliva. The concentrations of SCFAs and their relative distribution in saliva from five healthy subjects are shown in Table III. The concentrations were about seven time higher in the saliva than in the duodenal aspirates (for typical chromatograms see Fig. 2A).

Determination of SCFAs in human urine. Fig. 2B shows a typical chromatogram of a 24-h urine received from a healthy volunteer. 24-h Urine acetate excretions were very low (56.8 ± 14.1 μmol). These values are similar to those obtained by Pomare *et al.* [13] using GC with FID.

Determination of plasma SCFAs. Fig. 2C shows a typical chromatogram of plasma from a healthy subject. No significant amounts of butyrate, isobutyrate, valerate, isovalarate or hexonate were detected in venous blood, which is compatible with a high colonic metabolic trapping and/or a high hepatic clearance of these compounds. Only low amounts of formate, acetate and propionate were detectable. The average plasma concentrations of ten fasting subjects were 48.8 ± 3.4 μM for formate, 57.8 ± 8.4 μM for acetate and 28.8 ± 4.4 μM for propionate. These values are similiar to those obtained by Flemming and Rodriguez [15], but are in contrast to Tollinger *et al.* [20] and Pomare *et al.* [13], who, using GC with FID, detected only acetate in venous blood.

Determination of SCFAs in tissue of human colon and colon of the rat. The concentrations of SCFAs and their relative distribution in tissue of colon ascendens from rats and humans are quite similar. In both species, acetic acid (26.7 μmol/g protein *versus* 27.5 μmol/g *versus* protein) and isovaleric acid are the main SCFAs (198.7 μmol/g protein *versus* 43.1 μmol/g protein). Only traces of butyric and propionic acid are detectable, which documents their high metabolic trapping in this kind of tissue (for typical chromatograms see Fig. 2D).

CONCLUSION

A highly sensitive and reproducible but also simple HPLC–UV method has been developed for simultaneous and rapid determination of SCFAs in different biological materials, such as tissue, faeces, saliva, duodenal secretions, urine and plasma. After sample preparation by simplified vacuum transfer, direct injection of the alkaline phase without further handling allows for the detection of the SCFAs without any derivatization, giving results in less than 35 min. Compared with GC this method shows similar or better reproducibility. Additionally, it has proved to be efficient and durable, since we were able to make more than 1000 analyses on a single column over a period of three months. Therefore, if connected to an autosampler, this method easily can be applied for routine analysis in veterinary and clinical research and practice.

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