Assessment of the capabilities of capillary zone electrophoresis for the determination of hippuric and orotic acid in whey

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ABSTRACT

A rapid method was developed for the simultaneous determination of hippuric and orotic acid in rennet whey by capillary zone electrophoresis using an uncoated capillary utilizing a 0.04 M amino-2-methyl-1,3-propanediol (AMPD)–N,N-bis(2-hydroxyethyl) glycine (BICINE) buffer (pH 8.8) with UV detection at 254 and 280 nm. Whey proteins were removed by ultrafiltration. The method was evaluated for external, internal and standard addition procedures for both peak areas and peak heights. The use of an internal standard (sorbic acid) eliminated injection errors and gave, when applied to peak areas, the same levels for hippuric and orotic acid in those obtained with high-performance liquid chromatography. Relative standard deviations were 1–2%. Peak heights gave erratic results owing to sample matrix effects on peak widths.

INTRODUCTION

Milk and milk products contain small amounts of hippuric and orotic acid, and milk is the main source of these components in the human diet. In addition to their possible physiological properties [1], these components are especially of interest in the processing of liquid whey to give several products where the presence of these components may or may not be desirable at low concentrations. For instance, the production of pharmaceutical-grade lactose requires that the UV absorbance at 280 nm of the final product should meet a specific criterion as described in the Dutch Pharmacopoeia [2]. Orotic acid has an absorption maximum near 280 nm and its presence in lactose could, therefore, among other

components be significant for the suitability of lactose to be classified as pharmaceutical grade. The determination of orotic acid in milk bread permits the calculation of non-fat milk solids [3]. Current methods for the determination of the title compounds are based on reversed-phase high-performance liquid chromatography (HPLC) [3–6].

Capillary zone electrophoresis (CZE) in principle offers a high separation power, which can result in short analysis times. Recently, Goodall *et al.* [7] reviewed the quantitative aspects of CZE. In order to assess the possibilities of this technique in the field of dairy research, we have developed a CZE method to determine hippuric and orotic acid in whey.

EXPERIMENTAL

Reagents and chemicals

Buffer A, used for CZE, consisted of a 0.04 M solution of 2-amino-2-methyl-1,3-propanediol (AMPD) (Fluka, Buchs, Switzerland) titrated to pH 8.8 with 1 M N,N-bis(2-hydroxyethyl)glycine (BICINE) (Fluka). For sample preparation 0.02 M

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AMPD–BICINE (pH 8.8) was prepared (buffer B).

The calibration sample for CZE was prepared by weighing about 10 mg of orotic acid (BDH, Poole, UK) and 6 mg of hippuric acid (Sigma, St. Louis, MO, USA) and dissolving them together in 200 ml of buffer B. A 10-ml volume of this solution was diluted to 25 ml with buffer B after the addition of 50 μl of the internal standard solution of sorbic acid (50 mg/ml in buffer B) (Merck, Darmstadt, Germany) for detection at 254 nm and 625 μl for detection at 280 nm. Appropriate dilutions were prepared in order to obtain the calibration graph.

The calibration solution used with HPLC consisted of weighed amounts of about 22 mg of orotic acid and 8 mg of hippuric acid in 100 ml of the HPLC eluent and was diluted tenfold with the HPLC eluent.

Capillary zone electrophoresis

Electromigration was carried out with a Beckman P/ACE System 2000 controlled with a Laser 386/2 computer with Beckman P/ACE v. 1.50 software in combination with an uncoated fused-silica capillary (50 cm × 75 μm I.D.) fitted in a cartridge. Migrations were run at 25°C and the voltage across the capillary was maintained at 25 kV, with ground at the detector side. Injections were carried out by pressure (10 s). After each separation the capillary was flushed with 0.1 *M* sodium hydroxide solution for 18 s, followed by water (1 min) and buffer A (2 min). Detection was performed at 254 or 280 nm.

Peak areas and peak heights were obtained from the same raw data after processing with Caesar for Windows software (v. 1.0, B*Wise, Geleen, Netherlands). The capillary was blown dry with nitrogen on storage.

Liquid chromatography

Separations were carried out with a Model 6000A pump (Millipore–Waters, Milford, MA, USA), set at 0.6 ml/min, in combination with a Model ISS-100 automatic sample injector (25-μl injection) (Perkin Elmer, Überlingen, Germany). The column (HPX-87H; BioRad Labs., Richmond, CA, USA) was maintained at 30°C in an oven (CTO-2A; Shimadzu, Kyoto, Japan). The eluent was 0.005 *M* sulphuric acid, which was filtered prior to use. The solutes were detected with an Lambda Max Model 481 UV detector (Millipore–Waters) operated at 254 or 280 nm. The chromatograms were integrated with a Model SP4200 integrator (Spectra-Physics) and quantification was based on peak heights.

Sample treatment

For CZE analysis, 100 μl (detection at 254 nm) or 1250 μl (detection at 280 nm) of internal standard solution were added to a weighed amount of rennet whey (*ca.* 25 g) and the volume was made up to 50 ml with buffer A. This solution (10 ml) was diluted to 25 ml with buffer B. For standard addition 3, 5 and 10 ml of standard solution were added before the addition of buffer B. About 2 ml of the solution
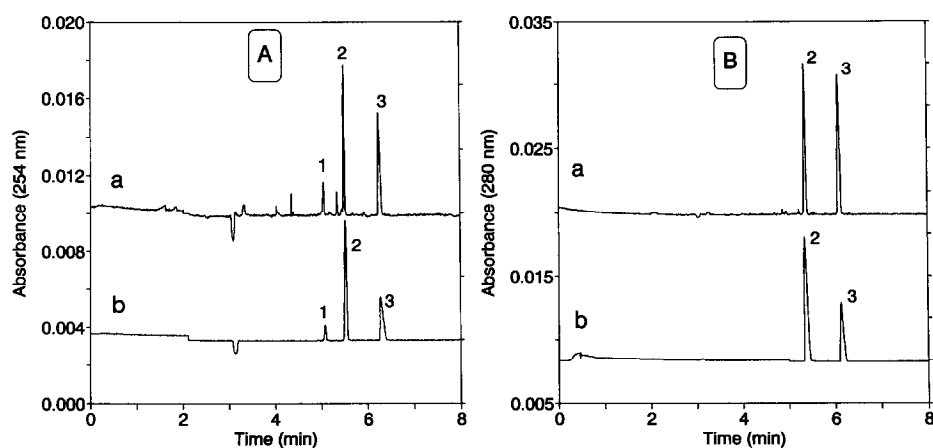

Fig. 1. Electropherogram of (1) hippuric, (2) sorbic and (3) orotic acid (a) in ultrafiltered rennet whey and (b) in a standard solution with detection at (A) 254 and (B) 280 nm. Buffer, AMPD–BICINE (pH 8.8)–0.04 *M* AMPD; voltage, 25 kV; detector side grounded.

obtained were ultrafiltered (LGC, modified cellulose, MW cut-off 10 000; Millipore). The first few drops were discarded and about 1 ml was collected for CZE analysis. About 30 μl of this solution were placed in a micro-insert vial.

For HPLC analysis, about 12.5 g of a weighed amount of rennet whey was diluted to 25 ml with 1 M perchloric acid and after standing for 1 h, 4 ml of this solution were filtered (MillexSLGV, Millipore, Molsheim, France) and the filtrate was used for analysis.

RESULTS AND DISCUSSION

Several buffers were investigated for the separation of hippuric and orotic acid in whey. In general we considered only so-called "super buffers", with good anion and cation buffering capacity and low conductivity. With detection at 214 nm there were in many instances interferences between matrix components and the peaks of the acids to be determined. In general, with most buffers satisfactory results were obtained when use was made of detection at 254 or 280 nm. An AMPD–BICINE (0.04 M, pH 8.8) buffer was chosen for further experiments on quantification. Sorbic acid proved to be a suitable internal standard, taking into account its migration position and its UV absorption at 254 and 280 nm
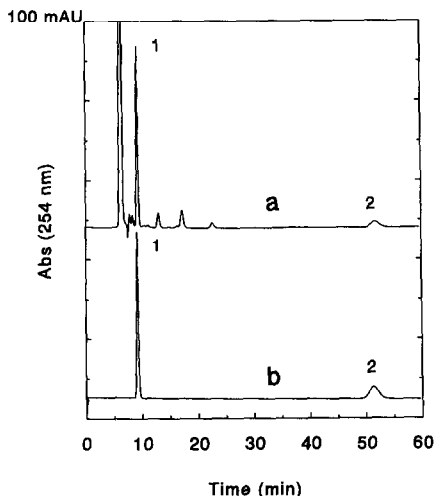
TABLE I

RELATIVE STANDARD DEVIATIONS OF THE RESPONSE FACTORS, BASED ON PEAK AREAS, OBTAINED AFTER FIVE CALIBRATIONS OF HIPPURIC ACID AND OROTIC ACID AT DETECTION WAVELENGTHS OF 254 nm AND 280 nm, CALCULATED WITH AND WITHOUT THE INTERNAL STANDARD

Detection wavelength (nm)	Internal/ external standard[a]	Relative standard deviation ($n = 5$) (%)	
		Hippuric acid	Orotic acid
254	I.S.	0.51	0.21
254	E.S.	6.8	6.8
280	I.S.	–	0.39
280	E.S.	–	1.9

[a] I.S. = calculated with internal standard (sorbic acid); E.S. = calculated without internal standard.

(Fig. 1). The HPLC separation is shown in Fig. 2 for the standards and whey.

The relative standard deviation (R.S.D.) of the response factors obtained with the calibration sample is given in Table I. At 280 nm the UV absorption of hippuric acid is negligible, so no response factors were obtained. A comparison of the results obtained for the external standard method with those for the internal standard method reveals that the precision of injection is sometimes poor. Errors
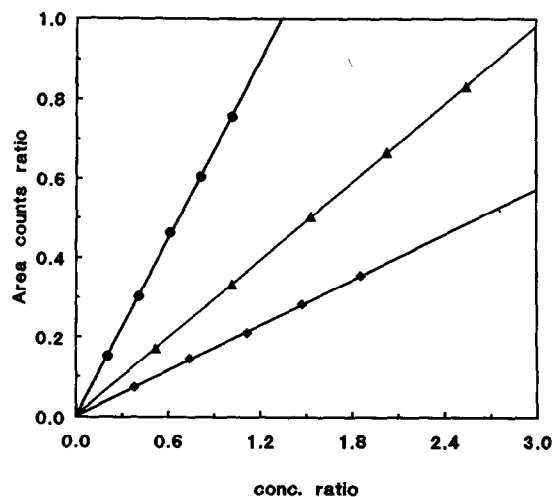

Fig. 2. Chromatogram of (1) hippuric and (2) orotic acid (a) in rennet whey and (b) in a standard solution. Column, HPX-87H at 30°C; eluent, 0.005 M sulphuric acid; flow-rate, 0.6 ml/min; detection at 254 nm.

Fig. 3. Calibration graphs obtained with sorbic acid as the internal standard and detection at 254 nm for ($\blacklozenge$) hippuric and ($\bullet$) orotic acid and detection at 280 nm for ($\blacktriangle$) orotic acid.

TABLE II

95% CONFIDENCE LIMITS FOR PREDICTED ANALYTE CONTENTS AT MEAN AREA COUNTS CORRESPONDING TO THE LOWER, MIDDLE AND UPPER PARTS OF THE CONCENTRATION RANGE USED FOR CALIBRATION

Ranges: hippuric acid, 2.85–28.5 μg/ml; orotic acid, 4.94–49.4 μg/ml.

Analyte	Detection wavelength (nm)	95% Concentration limit[a]					
		L (μg/ml)	M (μg/ml)	H (μg/ml)	L (%)	M (%)	H (%)
Hippuric acid	254	0.53	0.38	0.60	19	3.3	2.1
Orotic acid	254	0.96	0.67	1.13	17	3.4	2.3
Orotic acid	280	1.20	0.82	1.37	23	4.3	2.8

[a] L, M and H denote lower, middle and upper part of the calibration range, respectively. Calculations were performed with the Program RRGRAPH (version 4.52, Stichting Reactor Research, Delft University Press, 1990).

due to evaporation of solvent were expected to be small, because large volumes (4 ml) were used in the sample vials.

Fig. 3 shows the calibration graphs obtained at 254 and 280 nm. For both acids good linearity was observed. The offset, obtained after linear regression, was in general significantly different from zero but small. Calculations of the accuracy of the analytical results by using the regression equation are given in Table II. For the middle and higher parts of

TABLE III

AVERAGE RESULTS AND RELATIVE STANDARD DEVIATIONS FOR THE DETERMINATION OF HIPPURIC AND OROTIC ACID IN RENNET WHEY ($n = 5$) OBTAINED BY CZE IN COMBINATION WITH EXTERNAL, INTERNAL AND STANDARD ADDITION QUANTIFICATION AND WITH HPLC AS A REFERENCE METHOD

Solute	Quantification[a]	Concentration (mg/kg)		R.S.D. (%)	
		Peak area	Peak height	Peak area	Peak height
Hippuric acid[b]	E.S.	23.1	38.3	11	11
	I.S.	22.6	19.8	1.7	3.9
	S.A.i.	22.4	25.1	3.8	15
	S.A.m.	22.4	25.4	1.8	4.4
	HPLC	–	22.0	–	1.7
Orotic acid[b]	E.S.	75.5	111.6	10	5.8
	I.S.	74.0	58.0	0.9	3.0
	S.A.i.	74.3	172.3	2.4	23
	S.A.m.	74.5	177.0	1.0	3.3
	HPLC	–	74.9	–	0.3
Orotic acid[c]	E.S.	79.5	184.3	8.0	7.1
	I.S.	74.0	91.8	2.0	2.7
	S.A.i.	75.6	169.1	4.0	9.2
	S.A.m.	75.6	168.9	1.9	2.9
	HPLC	–	74.6	–	0.3

[a] E.S. = external standard; I.S. = internal standard; S.A.i. = standard addition, calibrated by linear regression of the three concentration levels for each sample; S.A.m. = standard addition, calibrated by linear regression of the average of three concentration levels of the five samples analysed; HPLC, average result obtained after five determinations with an HPX-87H column.
[b] Detection at 254 nm for CZE and HPLC.
[c] Detection at 280 nm for CZE and HPLC.

the calibration line acceptable results were obtained. On the basis of these observations, in practice one can obtain higher accuracy by using an average calibration (*e.g.*, $n = 5$) at a single concentration level, comparable to that expected for the samples. This approach was used in addition to standard addition to determine the little components in rennet whey.

The results obtained for rennet whey are given in Table III. Measurement of peak areas, irrespective of the method of quantification, in all instances gave values that did not differ statistically significantly (99% confidence) from those obtained with HPLC. This means that both CZE and HPLC are devoid of systematic errors in the determination of hippuric and orotic acid in whey, because both techniques are based on entirely different principles. Moreover, for orotic acid the results were obtained at two different wavelengths, 254 and 280 nm. The repeatabilities, expressed as relative standard deviations (R.S.D.), ranged from 0.9% to 2.0% for the internal standard (I.S.) and the standard addition method with averaged response factors (S.A.m.), respectively. The external standard (E.S.) method yielded a higher R.S.D. similar to that obtained with the standards. For standard addition with calibration at three levels (S.A.i.), higher R.S.D. values were obtained. In this instance the response factor was calculated after three determinations, whereas for the I.S. methods the average of five and for the S.A.m. method the averages of five determinations at three concentration levels were used.

Peak-height measurements gave erroneous results, caused by sample matrix effects on the peak width. These effects were different for each of the acids, including sorbic acid (Fig. 1). An example is the result obtained for orotic acid with the I.S. method: at 254 nm 58 mg/kg and at 280 nm 92 mg/kg were obtained. The only difference in the sample matrix is the concentration of the internal standard sorbic acid, which is 12.5 times higher if detection at 280 nm is used, compared with detection at 254 nm. In general, one can conclude that stacking of components occurs to a different extent for each analyte and that it is highly dependent on the sample matrix. Consequently, peak height is not a good parameter for quantification, unless one can rigorously control the sample matrix.

Migration of the electrophoretic zones occurs with different speeds, and consequently the peak widths increase with increasing migration times. Peaks areas can be normalized by division by their migration times [8]. Differences between the migration times of analytes in standards and in actual samples are sometimes observed. A constant difference for the migration times of the analytes in the standard and in the sample indicates a deviation of the electroosmotic flow during a short time (probably due to sample matrix effects), after which it regains its original value. In this instance normalization of peak areas is not allowed. If there is a constant relative difference, however, then the electroosmotic flow during the whole separation is different from its original value. Normalization of peak areas is then allowed and might improve the analytical precision.

We obtained R.S.D. values for the migration times of the acids in the range 0.9–1.4%, which was caused mainly by a gradual decrease in the migration times. The differences between the migration times of the analytes in the standard and in the sample were smaller than 0.1 min. Application of normalized areas resulted in almost the same average values and R.S.D.s for hippuric and orotic acid.

CONCLUSION

The determination of hippuric and orotic acid in whey by CZE is almost ten times faster than with HPLC. The repeatabilities are slightly lower (R.S.D. 0.9–2%) than those with HPLC (R.S.D. 0.3–1.7%), provided that use is made of an internal standard (sorbic acid). The levels found do not differ significantly from those obtained by HPLC.

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