Note

Reversed-phase ion-pair chromatography of some biologically important carboxylic acids as 2-nitrophenylhydrazides

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The direct microanalysis of a mixture of carboxylic acids, such as mono-, di-, tri- and hydroxy-carboxylic acids, which occur in biological materials, is difficult because the carboxyl functional group is only weakly chromophoric. In high-performance liquid chromatographic (HPLC) analysis, carboxylic acids are commonly monitored by refractive index or ultraviolet detection at about 210 nm, but these methods are not specific and have poor sensitivity.

To achieve the sensitive separation and detection of mixtures of the carboxylic acids, pre-column labelling reagents[1–8] or post-column reactions[9–12] have been developed. The pre-column labelling methods, however, were not satisfactory, because none of them could separate a mixture of mono-, poly- and hydroxy-carboxylic acids on the same chromatogram, and the post-column labelling methods also were not always satisfactory with respect to sensitivity, resolution and analysis time.

In previous work, both short- and long-chain fatty acids were found to react sensitively with 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl) to give acid hydrazides, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC · HCl) as a coupling agent, and were separated by reversed-phase HPLC[13]. This paper demonstrates the extension of the labelling technique to some biologically important di-, tri- and hydroxycarboxylic acids, and the separation of nanogram amounts of the mixture of the acid hydrazides using a reversed-phase ion-pair chromatographic (IPC) system.

EXPERIMENTAL

The carboxylic acids were converted into their hydrazide derivatives as described previously[13] and were submitted to HPLC. All chemicals used were of analytical-reagent grade and were obtained from commercial sources.

Chromatographic analyses were performed with a Shimadzu Model LC-5A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with a Shimadzu Model SPD-1 variable-wavelength UV–visible detector, which was set to monitor the absorbance at 400 nm. Chromatograms were recorded with a multi-pen recorder (Rikadenki Kogyo, Tokyo, Japan). The separation was performed with a C_8 reversed-phase column (250 × 4.6 mm I.D.) packed with YMC-C8 (particle size 5 μm) obtained from Yamamura Chemical Research Institute (Kyoto, Japan). The column temperature was maintained at 50°C.

All analyses were carried out isocratically using methanol–phosphate buffer as the eluent at a flow-rate of 1.2 ml/min. The pH was adjusted to the desired value by mixing methanol–0.005 M KH_2PO_4 with methanol–0.005 M Na_2HPO_4 and then dissolving counter ions at a concentration of 0.005 M. The counter ions studied were tetramethylammonium (TMA), tetraethylammonium (TEA) and tetra-n-propylammonium (TPA) (as bromides). The solvents were filtered through Millipore filters (pore size 0.65 μm) (Millipore, Bedford, MA, U.S.A.) and degassed with a Sonifer B-12 (Branson Sonic, CT, U.S.A.) before use.

The absorption spectra of the carboxylic acid hydrazides were measured with a Hitachi Model 340 spectrophotometer recorder (Hitachi, Tokyo, Japan).

RESULTS AND DISCUSSION

The derivatization procedure described previously[13] was applied to a number of acids representing some biologically important classes of carboxylates. Dicarboxylic, hydroxydicarboxylic and citric acids gave acidic acid hydrazides under the coupling conditions used.

Table I gives the visible absorbance peak maxima and the molar absorptivities of sixteen carboxylic acid hydrazides in basic media. The peak maxima and the molar absorptivities varied slightly with the type of carboxylic acid, but all of the acid hydrazides gave absorption maxima at 400 nm in the low pH region (pH < 8.5)[13], and were more sensitively detectable by monitoring at this wavelength. In addition, these acid hydrazides were stable for at least 14 days when kept in the reaction mixture at room temperature.

The separation of the above carboxylic acid hydrazides was first attempted by reversed-phase HPLC, but it was difficult to obtain adequate retentions owing to the high polarities of the residual carboxyl function.

TABLE I

ABSORPTION MAXIMA AND MOLAR ABSORPTIVITIES OF CARBOXYLIC ACID HYDRA-ZIDES IN BASIC MEDIUM

Carboxylic acid	$\lambda_{max.}$ (nm)	Molar absorptivity $\pm$ S.D. (n = 3) (l mol^{-1} cm^{-1})
Citric acid	550	10313 $\pm$ 34
Tartaric acid	540	6565 $\pm$ 21
Malonic acid	550	4115 $\pm$ 25
Malic acid	550	6947 $\pm$ 32
Succinic acid	550	7240 $\pm$ 27
Glutalic acid	550	7880 $\pm$ 24
Methylmalonic acid	550	4436 $\pm$ 14
Fumaric acid	570	9886 $\pm$ 29
Adipic acid	550	14261 $\pm$ 35
Glycolic acid	540	6457 $\pm$ 21
L-Pyroglutamic acid	540	2860 $\pm$ 11
Formic acid	530	2790 $\pm$ 19
Lactic acid	540	4343 $\pm$ 22
Acetic acid	545	5151 $\pm$ 20
Maleic acid	560	7072 $\pm$ 26
Propionic acid	550	5285 $\pm$ 17

Reversed-phase ion-pair chromatography (IPC), in which a hydrophobic stationary phase and an aqueous buffer containing a low concentration of counter ion are used, allows the separation of both ionized and non-ionized components under the same chromatographic conditions. Some chromatographic properties were determined for HPLC analyses of the carboxylic acid hydrazides using a YMC-C8 column and methanol–phosphate buffer (25:75) containing quaternary ammonium ion as the eluent. The separation selectivity can be influenced by either the pH of the eluent or the nature of the counter ions. The pH of the eluent was chosen as 7.0. The acidic acid hydrazides ionize at pH 7.0 and the retentions will therefore depend on the nature of the ion pairs, and the lipophilic character of the counter ion affects their retention characteristics.

TABLE II

RETENTION TIMES OF CARBOXYLIC ACID HYDRAZIDES AND RELATIVE RETENTION TIMES WITH RESPECT TO GLYCOLIC ACID HYDRAZIDE ELUTED WITH DIFFERENT COUNTER IONS

Values given are retention times (min) with relative retention times in parentheses. Chromatographic conditions: column, YMC-C8 (5 μm), 250 × 4.6 mm I.D.; column temperature, 50°C; mobile phase, methanol–0.005 M KH$_2$PO$_4$–0.005 M Na$_2$HPO$_4$ (25:75) + 0.005 M counter ion, pH 7.0; flow-rate, 1.2 ml/min.

Carboxylic acid	Counter ion		
	TMA	TEA	TPA
Citric acid	2.66 (0.44)	3.00 (0.50)	6.24 (1.04)
Tartaric acid	3.78 (0.63)	4.20 (0.70)	6.37 (1.06)
Malonic acid	4.25 (0.71)	4.58 (0.76)	7.12 (1.18)
Malic acid	4.27 (0.71)	4.62 (0.77)	7.19 (1.20)
Succinic acid	4.44 (0.74)	4.88 (0.81)	7.46 (1.24)
Glutalic acid	5.03 (0.84)	5.45 (0.91)	8.94 (1.49)
Methylmalonic acid	5.05 (0.84)	5.52 (0.92)	8.97 (1.49)
Fumaric acid	5.11 (0.85)	5.58 (0.93)	10.17 (1.69)
Adipic acid	5.84 (0.98)	6.50 (1.08)	11.00 (1.83)
Glycolic acid	5.98 (1.00)	6.00 (1.00)	6.01 (1.00)
L-Pyroglutamic acid	7.12 (1.19)	7.08 (1.18)	7.09 (1.18)
Formic acid	7.44 (1.24)	7.46 (1.24)	7.45 (1.24)
Lactic acid	8.01 (1.34)	8.02 (1.34)	8.09 (1.35)
Acetic acid	8.35 (1.40)	8.40 (1.40)	8.43 (1.40)
Maleic acid	11.25 (1.98)	14.44 (2.41)	35.10 (5.84)
Propionic acid	13.46 (2.25)	13.54 (2.26)	13.58 (2.26)

The retention times and the relative retention times with respect to glycolic acid hydrazide obtained with TMA, TEA and TPA counter ions are given in Table II. An increase in the alkyl chain length of the counter ions produced a corresponding increase in the retention times of the acidic acid (*i.e.* di- or trivalent carboxylic acid) hydrazides, but mono-acid (*i.e.* monovalent carboxylic acid) hydrazides showed no change in retention time. The counter ions also had a significant effect on the separation selectivity of various pairs of mono-acid and acidic acid hydrazides such as glycolic–adipic, glycolic–citric and formic–succinic acid hydrazides. With TMA, the

glycolic–adipic acid hydrazide pair remained close together and unseparated. When TEA or TPA was used instead of TMA, they were separated. Both the glycolic–citric and formic–succinic acid hydrazide pairs were separated with TMA or TEA, but were unseparated when TPA was used. The acidic glutaric-fumaric acid hydrazide pair was separated only with TPA, but this counter ion markedly increased the overall retention time. Consequently, it was confirmed that thirteen kinds of acid hydrazides were best separated with TEA. However, the malonic–malic and glutaric–methyl-malonic acid hydrazide pairs had similar retention times in all instances and another elution system would be necessary in order to improve their resolution.

A typical chromatogram obtained with TEA as the counter ion is shown in Fig. 1 and indicates the good chromatographic properties of the hydrazides, with short retention times and symmetrical peaks. The peaks of citric, tartaric, malonic (malic), succinic, glutaric (metylmalonic or fumaric), glycolic, adipic, L-pyroglutamic, formic, lactic, acetic, propionic and maleic acid hydrazides were clearly separated from each other within 15 min.
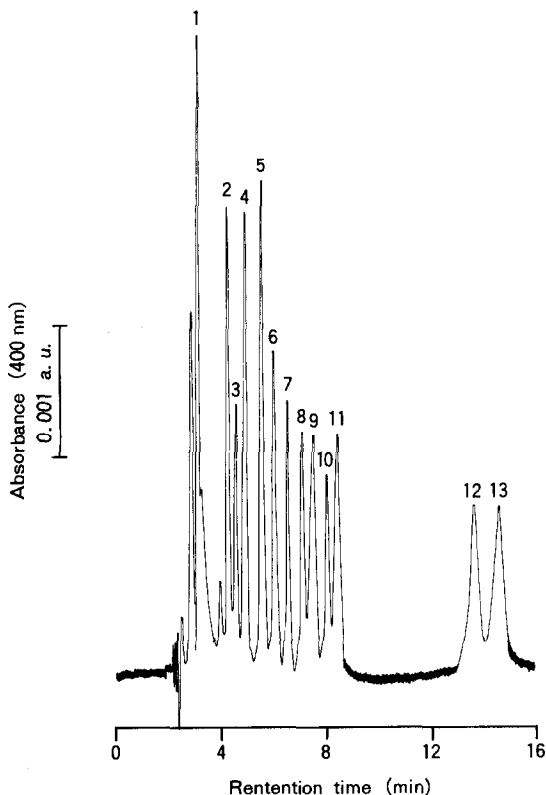

Fig. 1. Reversed-phase IPC of carboxylic acid hydrazides using TEA bromide. The system indicated is defined in Table II, and the amounts of sample varied from 150 to 300 pmol of each acid. Peaks: 1 = citric; 2 = tartaric; 3 = malonic (malic); 4 = succinic; 5 = glutaric (methylmalonic or fumaric); 6 = glycolic; 7 = adipic; 8 = L-pyroglutamic; 9 = formic; 10 = lactic; 11 = acetic; 12 = propionic; 13 = maleic acid hydrazide.
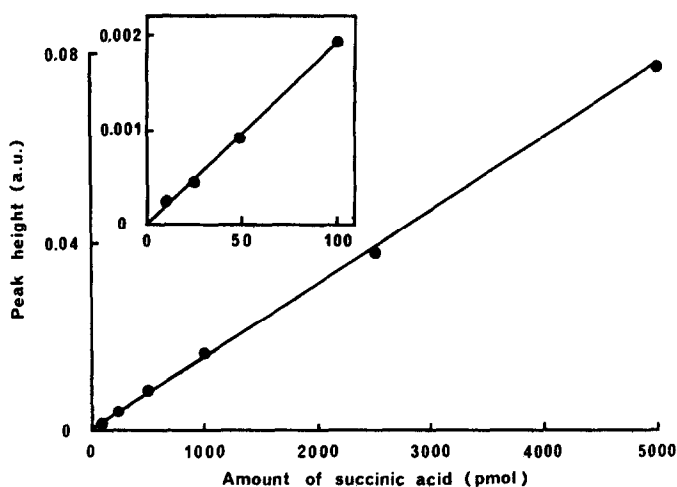
Fig. 2. Calibration graph for succinic acid.

To construct a calibration graph, known concentrations of succinic acid were converted into the hydrazide and an aliquot was injected into the chromatograph. The graph of peak height *versus* concentration of the acid is linear at least in the range 10 pmol–5 nmol per injection and passes through the origin, as shown in Fig. 2. The coefficient of variation was 1.5% ($n = 7$) for 100 pmol per injection and the limit of detection with a signal-to-noise ratio of 3:1 was 10 pmol per injection.

In conclusion, the proposed method has advantages with respect to resolution, analysis time and sensitivity compared with previously published methods, and may be used for the routine analysis of carboxylic acids in various fields.

ACKNOWLEDGEMENT

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