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Simple cation-exchange high-performance liquid chromatography optimized to the measurement of metabolites in the effluents from perfused rat livers using refractive index and ultraviolet detectors

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ABSTRACT

A high-performance liquid chromatographic method designed to analyse effluents from perfused organs is described. In the case of rat liver, compounds released by the liver are readily separated and quantitated, using a strong cation exchanger (Aminex HPX 87H), two detectors connected in series (ultraviolet detector at 210 nm and refractive index detector), and by optimizing the concentration of sulphuric acid in the mobile phase. Chromatographic conditions described in the present work enable the quantitation, in a single run, of metabolites derived from the tricarboxylic acid cycle, glycolysis, ketogenesis, adenine nucleotides catabolism and ethanol oxidation. The advantage of this method stems from its ease of implementation, sensitivity and flexibility.

INTRODUCTION

High-performance liquid chromatography (HPLC) is now widely used in biomedical and pharmacological applications. For example, metabolic disorders in humans are readily detected by HPLC analysis of physiological samples, such as urine or plasma [1–4]. Reports dealing with insect haemolymph [5], perchloric acid extracts from rat liver [6] or rabbit renal medulla [7], chloroform–methanol extracts from rat brain or liver [8], microdialysis samples from rat organs [9] or media obtained from cell cultures [10,11] have recently been presented. Ion-moderated partition HPLC [12] with strong cation exchangers seems to be well suited for a quantitative analysis of various metabolites in a physiological sample [13,14]. Although reversed-phase [9,15,16] or anion-exchange [6] chromatography can also be used, cation exchange permits efficient and simple separation of organic (carboxylic) acids [1,2,5,17–19], carbohydrates [11,20–22] and alcohols [20,21,23] in various biological fluids.

In the course of studies we have conducted by nuclear magnetic resonance

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(NMR) spectroscopy [24–26] on ethanol-induced metabolic perturbations in perfused rat liver, liver effluents have been analysed by HPLC to allow the simultaneous collection of information from the parenchyma and from compounds released in the perfusate. HPLC analysis has been performed with a cation exchanger and double detection (refractive index and UV at 210 nm). Combined detection increases the detection sensitivity of compounds such as pyruvate, allantoin or fumarate, and allows the release of urate to be monitored. Hepatic release of metabolites arising from the glycolytic and related pathways, the tricarboxylic acid cycle, ketogenesis, adenine nucleotide catabolism and ethanol oxidation has also been monitored and quantitated.

EXPERIMENTAL

High-performance liquid chromatography

Analyses were carried out with a LKB chromatographic system (LKB, Bromma, Sweden) consisting of an HPLC pump (Model 2150), a variable-wavelength detector (Model 2151) and a differential refractometer detector (Model 2142) connected in series, and a Rheodyne 7125 injector valve fitted with an automated trigger for data acquisition. The column was a cation-exchange Aminex HPX 87H (300 mm × 7.8 mm I.D., 9 μm particle size, BioRad, Richmond, CA, U.S.A.) protected by a cation-H^+ guard column cartridge (40 mm × 4.6 mm I.D., BioRad). Data were acquired and chromatograms integrated on both channels (UV at 210 nm and refractive index) using a Kontron PC integration software package (Kontron Instruments, Munich, Germany).

The mobile phase consisted of 1.25 m*M* sulphuric acid (Suprapur, Merck, Darmstadt, Germany), daily diluted from a 1 *M* stock solution in Milli-Q water (Millipore, Molsheim, France) and filtered through a 0.22-μm filter. For identification purposes and purity assessment, concentrations of sulphuric acid in the mobile phase were varied between 0.25 and 5 m*M*. After thorough helium degassing and equilibration at room temperature, the mobile phase was delivered at a flow-rate of 0.6 ml/min. In order to avoid thermal drifts between the reference and the sample cells of the refractometer, helium-degassed water was allowed to flow by force of gravity through the reference cell. All standard solutions of compounds were prepared from analytical-grade chemicals (Sigma, St. Louis, MO, U.S.A.).

Liver perfusion and effluent collection

Male Wistar rats (240–280 g) were used as liver donors. The livers were perfused anterogradely in a non-recirculating mode as previously described [24]. The perfusion medium was a Krebs–Henseleit buffer made up with 120 m*M* NaCl, 24 m*M* $NaHCO_3$, 0.48 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$ and 1.3 m*M* $CaCl_2$ dissolved in deionized water. Effluents from livers were collected in disposable tubes, immediately frozen and kept at −80°C. Prior to injection for

HPLC analysis, the effluents were gently thawed at room temperature and filtered through a 0.45-μm filter.

RESULTS

Effect of pH on chromatographic separation

In order to define optimal conditions of chromatographic separation at room

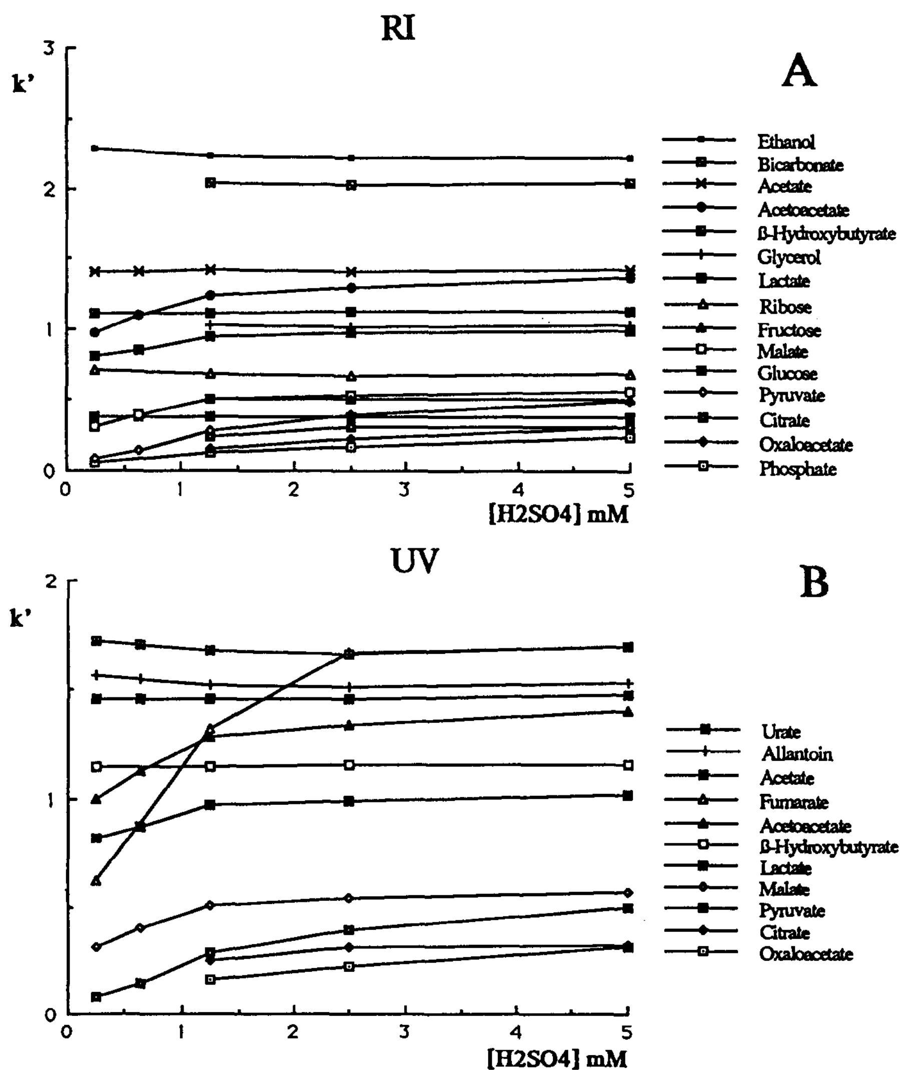

Fig. 1. Variation of the capacity factors (k') of some standard compounds as a function of the concentration of sulphuric acid in the mobile phase. (A) Refractive index detection; (B) UV detection at 210 nm. The sulphuric acid concentration was varied between 0.25 and 5 mM in the mobile phase, and k' was calculated for each compound. Flow-rate, 0.6 ml/min.

temperature, the concentration of sulphuric acid in the mobile phase was varied between 0.25 and 5 m*M*. The resulting variations of capacity factors (k') for some compounds detected by refractometry (Fig. 1A) and/or UV absorbance at 210 nm (Fig. 1B) are presented. Optimization of the chromatographic conditions relied on the differential behaviour of the compounds towards the acidity of the mobile phase. The capacity factors of carbohydrates and of organic acids with the highest acid dissociation constants (pK_a), *e.g.* β-hydroxybutyrate, acetate and urate, were fairly constant. Conversely, retention of milder organic acids was dependent on the pK_a of carboxylic acid functions, as previously described [18,23]. Concentrations of sulphuric acid lower than 0.25 m*M* were avoided (i) owing to their deleterious effects on the column matrix and (ii) because they did not afford adequate resolution between some of the metabolites and the compounds eluted in the void volume.

Figs. 2 and 3 display chromatograms from two different standard solutions, obtained with 5 m*M* (A) or 1.25 m*M* (B) sulphuric acid. The compounds selected in the solutions derive from the tricarboxylic acid cycle (oxaloacetate, citrate and fumarate), the glycolytic and related pathways (glucose, fructose, glycerol, pyruvate and lactate), ketogenesis (β-hydroxybutyrate and acetoacetate), adenine nucleotide catabolism (allantoin and urate) and salts from the Krebs medium (inor-

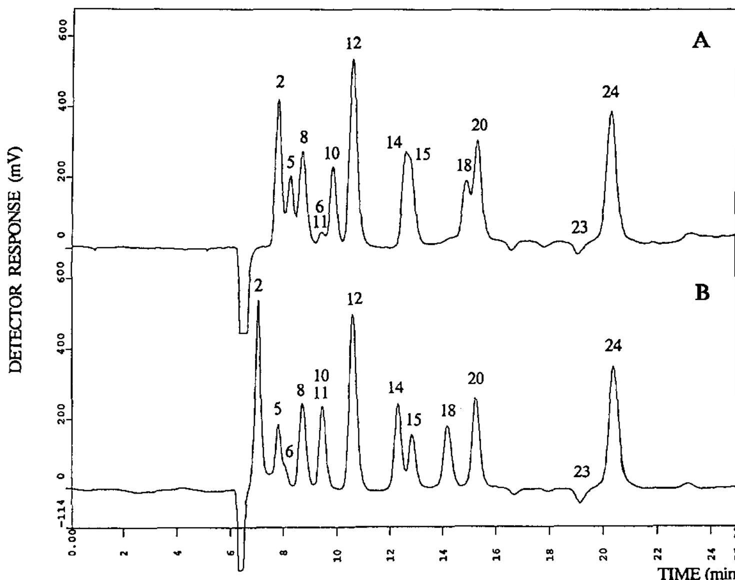

Fig. 2. Chromatograms of a standard solution detected by refractometry. (A) Mobile phase 5 m*M* sulphuric acid; (B) mobile phase 1.25 m*M* sulphuric acid. Peaks: 2 = inorganic phosphate (50 nmol); 5 = citrate (16 nmol); 6 = pyruvate (4.5 nmol); 8 = glucose (16 nmol); 10 = malate (20 nmol); 11 = fructose (1nmol); 12 = ribose (50 nmol); 14 = lactate (40 nmol); 15 = glycerol (4.5 nmol); 18 = acetoacetate (60 nmol); 20 = acetate (100 nmol); 23 = bicarbonate (80 nmol); 24 = ethanol (260 nmol).

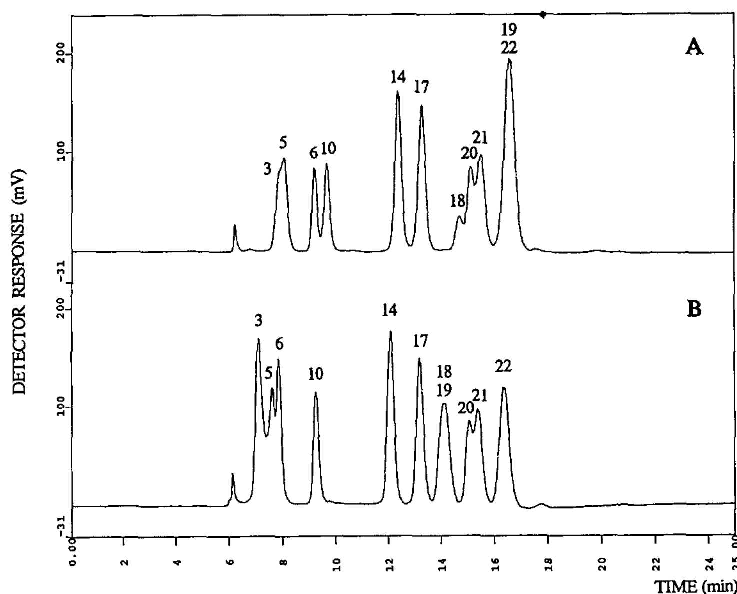

Fig. 3. Chromatograms of a standard solution detected by UV at 210 nm. (A) Mobile phase 5 m*M* sulphuric acid; (B) mobile phase 1.25 m*M* sulphuric acid. Peaks: 3 = oxaloacetate (3 nmol); 5 = citrate (4 nmol); 6 = pyruvate (2 nmol); 10 = malate (20 nmol); 14 = lactate (50 nmol); 17 = β-hydroxybutyrate (100 nmol); 18 = acetoacetate (23 nmol); 19 = fumarate (0.27 nmol); 20 = acetate (110 nmol); 21 = allantoin (1.6 nmol); 22 = urate (1 nmol).

ganic phosphate and bicarbonate). The improvement of chromatographic resolution with 1.25 m*M* sulphuric acid, for some pairs of compounds poorly resolved at 5 m*M*, is illustrated in Fig. 2 (lactate/glycerol and acetoacetate/acetate pairs) and in Fig. 3 (fumarate/urate and oxaloacetate/citrate pairs). A concentration of 1.25 m*M* sulphuric acid (pH 2.63) was eventually chosen for the rest of this study, based on the consideration of the optimal resolution afforded for all compounds of interest.

Identification of peaks

Peaks were assigned by comparing (i) their capacity factors and (ii) the ratios of the response factors [UV detection at 210 nm/refractive index (RI) detection] with those of pure compounds. Capacity factors for the metabolites present in Figs. 2 and 3 and for several other compounds are reported in Table I, together with the ratios of the response factors (UV/RI). Advantage was taken of a great difference in the respective response factors in UV and RI detections to better quantify compounds from partially resolved peaks. For example, acetate was preferentially integrated on the RI channel, where the allantoin signal was very weak. These ratios allowed the homogeneity of peaks in chromatograms recorded on biological samples to be checked. The response factors of various compounds,

TABLE I

CAPACITY FACTORS, RATIOS OF RESPONSE FACTORS (UV/RI) AND RELATIVE RESPONSE FACTORS OF STANDARD COMPOUNDS

All parameters were measured under the following conditions: mobile phase, 1.25 m*M* sulphuric acid; flow-rate, 0.6 ml/min; room temperature; UV sensitivity, 0.02 a.u.f.s.; RI sensitivity, 2. a.u. For the calculation of k', t_0 = 6.13 and 6.30 min for the UV channel and the RI channel, respectively. Relative response factors are the area ratios for equimolar concentrations of a compound and lactate, for each channel.

Compound	Peak No.	Capacity factor		Ratio of response factors (UV/RI)	Relative response factor	
		UV	RI		UV	RI
cis-Aconitate	1	0.085	n.d.[a]	–	150	n.d.[a]
Phosphate	2	–	0.119	–	–	1.50
Oxaloacetate	3	0.161	0.157	3.3	17.2	1.78
α-Ketoglutarate	4	0.177	0.173	3.4	18.3	1.88
Citrate	5	0.248	0.242	0.44	3.67	2.88
Pyruvate	6	0.287	0.281	5.5	19.2	1.12
Isocitrate	7	0.291	0.285	0.33	2.67	2.78
Glucose	8	–	0.383	–	–	3.03
Galactose	9	–	0.468	–	–	9.93
Malate	10	0.511	0.499	0.34	1.86	1.89
Fructose	11	0.524	0.507	< 0.01	–	2.59
Ribose	12	0.701	0.681	< 0.01	–	2.45
Succinate	13	0.953	0.927	0.24	1.02	1.45
Lactate	14	0.974	0.948	0.35	1	1
Glycerol	15	–	1.033	–	–	12.6
Dihydroxyacetone	16	1.099	1.069	0.15	0.513	1.17
β-Hydroxybutyrate	17	1.151	1.121	0.19	0.754	1.34
Acetoacetate	18	1.284	1.248	0.60	2.21	1.27
Fumarate	19	1.315	1.279	34.3	216	2.17
Acetate	20	1.457	1.418	0.37	0.503	0.472
Allantoin	21	1.517	1.475	3.5	38.7	3.64
Urate	22	1.678	–	–	85.9	–
Bicarbonate	23	–	2.035	–	–	0.105
Ethanol	24	–	2.232	–	–	0.331

[a] Not detectable.

relative to lactate response factor, for both UV and RI detections are also presented in Table I, emphasizing the large range of sensitivity for the UV-absorbing compounds.

Linearity of the calibration curves

The calibration curves on both channels were determined over a wide range of concentrations, encompassing the physiological range for metabolites likely to be present in effluents from perfused rat liver (Table II). Linear relationships were

TABLE II

LINEARITY OF THE CALIBRATION CURVES, DETECTION LIMITS, AND RESPONSE FACTORS FOR SOME SELECTED COMPOUNDS

Response factors were measured under the following conditions: mobile phase, 1.25 m*M* sulphuric acid; flow-rate, 0.6 ml/min; room temperature, UV sensitivity, 0.02 a.u.f.s.; RI sensitivity, 2 a.u. Response factors are defined as the slopes of the variations of peaks areas *versus* injected amounts (mV min/nmol). The limits of detection on both channels were set for a minimal signal-to-noise ratio of *ca.* 5 on chromatograms.

Compound	Amount injected (nmol)	UV		RI		Detection limit[a] (n*M*)
		Response factor (mV min/nmol)	Correlation coefficient (r^2)	Response factor (mV min/nmol)	Correlation coefficient (r^2)	
Pyruvate	0.04–4	12.59	1.000	–	–	25
Glucose	1.25–40	–	–	5.76	0.999	1000
Malate	0.4–40	1.220	1.000	3.60	1.000	250
Lactate	1–100	0.656	1.000	1.90	1.000	500
β-Hydroxybutyrate	1–100	0.495	1.000	2.56	1.000	500
Acetoacetate	0.4–40	1.450	1.000	2.41	0.997	250
Acetate	1.5–50	0.330	1.000	0.89	1.000	1000
Allantoin	0.01–1	25.4	1.000	–	–	10
Urate	0.01–1	56.3	1.000	–	–	10
Ethanol	10–400	–	–	0.63	1.000	4000

[a] 200-μl loop.

measured over the whole range of concentrations with correlation coefficients (r^2) higher than 0.997. The relative standard deviations (R.S.D.) for response factors were less than 5% ($n = 25$). The detection limits of compounds, assuming a lowest signal-to-noise ratio of 5 on the chromatograms, are also presented in Table II; they compare favourably with results from previous studies [1,16].

HPLC analysis of perfused liver effluents

The sensitivity of UV detection, the resolution and the high information content of the chromatograms are illustrated in Fig. 4. A chromatogram of the effluent collected from a fed rat liver perfused with a Krebs–Henseleit medium is shown in Fig. 4A. The main peaks have been assigned to citrate, pyruvate, lactate, acetoacetate, allantoin and urate, and concentrations of these metabolites are reported in Table III (control). In the liver from fed rat, addition of 2 m*M* ethanol or 10 m*M* ethanol and 1 m*M* ribose to the perfusion medium leads to profound metabolic perturbations, as revealed by the chromatograms in Fig. 4B and C, respectively. Ethanol oxidation induces a large decrease in the hepatic release of pyruvate and lactate, the former reaching a very low, albeit quantifiable level (Table III). Allantoin and urate, which are end-products of adenine nucleo-

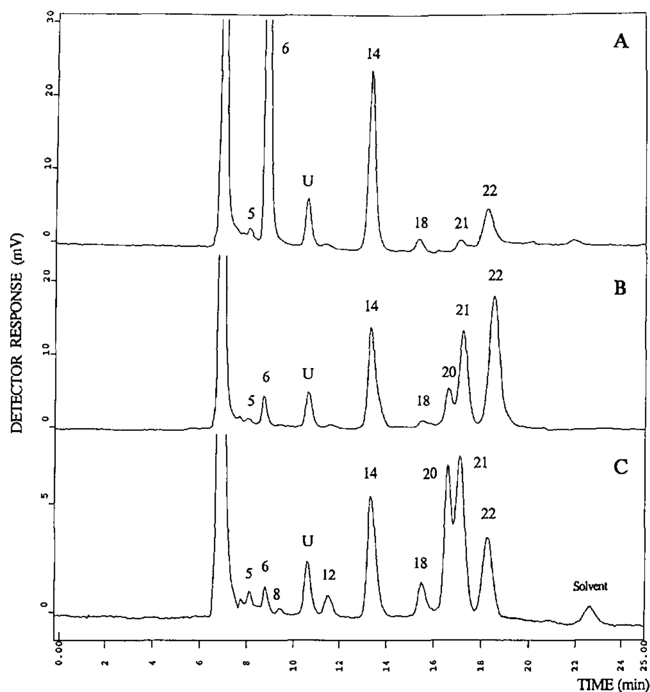

Fig. 4. Effect of ethanol on the chromatographic profile of the effluent of a perfused fed rat liver. (A) perfusion with a Krebs–Henseleit medium; (B) perfusion with a Krebs–Henseleit medium supplemented with 2 m*M* ethanol; (C) perfusion with a Krebs–Henseleit medium supplemented with 10 m*M* ethanol and 1 m*M* ribose. Detection, UV at 210 nm; mobile phase, 1.25 m*M* sulphuric acid; flow-rate, 0.6 ml/min. Peaks as listed in Table I. U = unknown compound.

TABLE III

ETHANOL-INDUCED MODIFICATIONS IN THE HEPATIC RELEASE OF METABOLITES IN THE EFFLUENTS FROM PERFUSED RAT LIVERS

Livers from fed rats were perfused as described under Experimental with a Krebs–Henseleit buffer (control). After a 30-min equilibration period, the medium was supplemented with 2 m*M* ethanol. Concentrations of metabolites in the effluents are reported as means ± standard deviations for ten experiments.

Metabolite	Concentrations (μM)	
	Control	2 m*M* Ethanol
Citrate	4.0 ± 0.037	n.d.[a]
Pyruvate	96.0 ± 9.57	3.78 ± 0.63
Glucose	428 ± 50	487 ± 39
Lactate	510 ± 45	320 ± 33
Acetoacetate	22 ± 2.65	n.d.[a]
Acetate	n.d.[a]	246 ± 22
Allantoin	1.257 ± 0.17	9.68 ± 1.38
Urate	2.44 ± 1.17	5.93 ± 1.65
Ethanol	n.d.[a]	1400 ± 59

[a] Not detectable.

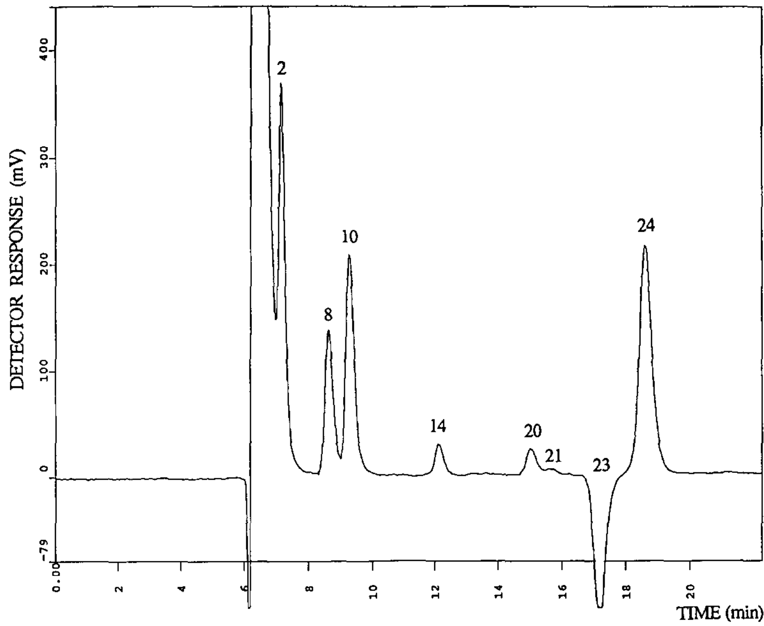

Fig. 5. Chromatogram of the effluent of a rat liver perfused with a Krebs–Henseleit medium supplemented with 1 mM malate and 10 mM ethanol. Detection, refractive index; mobile phase, 1.25 mM sulphuric acid; flow-rate, 0.6 ml/min. Peaks as listed in Table I.

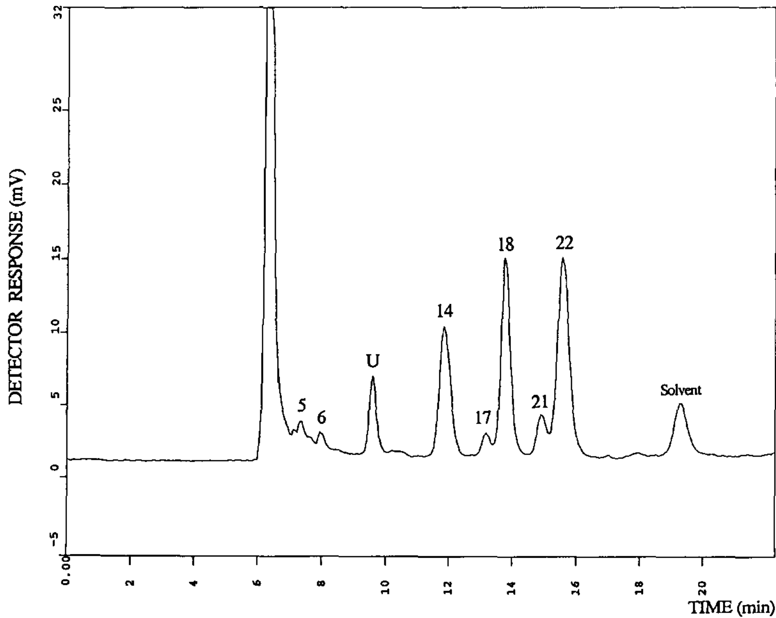

Fig. 6. Chromatogram of the effluent of a liver from a starved rat perfused with a Krebs–Henseleit medium. Detection, UV at 210 nm; mobile phase, 1.25 mM sulphuric acid; flow-rate, 0.6 ml/min. Peaks as listed in Table I. U = unknown compound.

tide catabolism in the rat, increase after ethanol perfusion. Finally, the oxidation of ethanol leads to hepatic release of acetate in the effluent (Table III).

The chromatogram (RI detection) of the effluent from a rat liver perfused with 1 m*M* malate and 10 m*M* ethanol is presented in Fig. 5. Apart from the peaks of inorganic phosphate and hydrogenocarbonate arising from the perfusion medium, glucose, malate, lactate, acetate and ethanol can be easily quantified. Acetate was preferentially integrated on the RI channel, owing to the weak refractive index of allantoin (Table I).

Metabolic modifications occurring during fasting are illustrated in Fig. 6. A decrease in glycolysis caused by a substrate depletion induced by fasting is balanced by activation of ketogenesis, as revealed by the ratios of lactate to β-hydroxybutyrate and of pyruvate to acetoacetate (Fig. 4A, Fig. 6).

DISCUSSION

The aim of this study was to establish a simple and time-saving method for the quantitative and kinetic analysis of various metabolites in the effluents from perfused organs. The purity and homogeneity of the peaks were carefully assessed using a double detection method based on refractometry and UV absorbance at 210 nm. This system allowed the ratios of peak areas for the two detectors for a standard compound to be calculated, and compared with ratios measured under the same chromatographic conditions for compounds in the effluents. For example, lactate peak integration was performed on the RI detection channel because of a slight contamination of the UV peak of lactate by an unknown compound released after ethanol perfusion of the liver. On the other hand, UV detection at 210 nm was required for compounds with low RI response factors such as pyruvate and urate.

Peak purities and assignments have also been confirmed based on the comparative chromatographic behaviour of pure standards and metabolites in the effluents as a function of the acidity in the mobile phase. An optimized resolution for all compounds of interest in this study has been established using 1.25 m*M* sulphuric acid (pH 2.63), although at this pH a shift of some metabolites towards the void volume of the column occurs (*e.g.* α-ketoglutarate).

In this study, optimization of chromatographic resolution was obtained by varying the acidity of the mobile phase, as already described [23]. Nevertheless, most sophisticated procedures require a change of temperature of the column [5,20], use two columns connected in series [27] or combine these two techniques [17] in order (i) to improve further the resolution efficiency with increasing temperatures or (ii) to enable the detection of compounds previously eluting in a volume close to the void volume of a single-column device.

A competitive advantage of this method rests in its simplicity of implementation. The isocratic elution allows the use of a refractive index detector and reduces the delay between two consecutive analyses. The samples did not require any

treatment before injection, except for a rapid filtration. Finally, the good resolution and the high signal-to-noise ratio of the chromatograms allowed the reliable quantitation of metabolites present at low concentrations in the effluents.

Ethanol oxidation induces profound modifications on the hepatic redox and phosphorylation states [25,28], which can be readily monitored on the HPLC profiles of the effluents. A large decrease in the hepatic release of lactate and pyruvate after ethanol perfusion (Fig. 4B, Table III) suggests an inhibition of glycolysis, probably at the level of glyceraldehyde 3-phosphate dehydrogenase [29], owing to the ethanol-induced accumulation of reduced nicotinamide-adenine dinucleotide (NADH) [26]. The increased release of uric acid and allantoin during ethanol perfusion stems from enhanced ATP hydrolysis and adenine nucleotide catabolism. Indeed, ethanol perfusion induces a large accumulation of glycero-3-phosphate in the hepatocytes from fed rats, hence trapping inorganic phosphate [24], which is an inhibitor of AMP deaminase [30]. Its depletion activates adenine nucleotide catabolism, allantoin being the end-product of this pathway in the rat [26].

Ethanol is oxidized to acetaldehyde and acetate, which can be exported from the hepatocytes [31]. Again, ethanol consumption and subsequent acetate release have been directly monitored by HPLC using RI detection.

The simple and sensitive chromatographic method described in this paper and developed for liver studies can be more generally applied to the follow-up of metabolic events in the effluents of any perfused organ preparation.

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