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ANALYTICAL ISOTACHOPHORESIS UTILIZING COMPUTER SIMULATION

II. ASSESSMENT OF OPTIMUM SEPARATION CONDITIONS FOR URINARY TRIFLUOROACETIC ACID METABOLIZED FROM ANAESTHETIC HALOTHANE

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SUMMARY

Experimentally determined optimum separation conditions for a metabolite of anaesthetic halothane in urine, trifluoroacetic acid, were assessed by means of computer simulation of the isotachophoretic steady-state. The simulation confirmed that urinary acids and trifluoroacetic acid can be separated in the limited pH range of 3.5—3.7 buffered by β-alanine, as far as the pH dependence of effective mobility is utilized. The separated fraction of the trifluoroacetic acid zone was identified by mass spectrometry. The simulated coefficient of the calibration curve agreed well with the observed value.

INTRODUCTION

Trifluoroacetic acid (TFA) is the main metabolite of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) which is a volatile anaesthetic widely used in clinical anaesthesia and has traditionally been considered an inert anaesthetic. However, hepatitis following halothane anaesthesia has been reported [1] and

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it was suggested that the toxicity of halothane may be associated with the metabolism of the anaesthetic [2]. Therefore, it is necessary to elucidate the metabolism of this drug. It is generally recognized that halothane is metabolized into TFA, F^-, and Br^- in urine together with CF_2CHCl and CF_3CH_2Cl in expiratory gas. Br^- is a natural component of the human body; therefore precise determination of Br^- metabolized from halothane is difficult. On the other hand, TFA is not present in the body and the total urinary excretion of TFA is the largest among the metabolites.

In a previous paper [3] we have shown that the isotachophoretic determination of TFA is more advantageous than previously described techniques such as paper chromatography [4], thin-layer chromatography [5], and gas chromatography [6]. Namely, the latter methods require complicated sample pretreatment, well trained technicians and long periods for analysis.

In the present paper, the experimentally determined optimum separation conditions and coefficient of the calibration curve for quantification of TFA were analysed by means of a computational method based on the simulation of isotachophoretic qualitative [7] and quantitative indices [8] to test the analytical utility of the present method in biochemical fields.

ASSESSMENT OF OPTIMUM SEPARATION CONDITIONS

To assay TFA by means of isotachophoresis, the optimum separation conditions have been assessed experimentally among the normal urinary acids, lactic acid, phosphoric acid, pyruvic acid, and the objective metabolites (TFA, F^-, and Br^-) [3].

The optimum electrolyte system found was as follows: leading electrolyte 0.01 *M* hydrochloric acid buffered by β-alanine (pH 3.5—3.8) and terminating electrolyte 0.01 *M* caproic acid. A surfactant Triton X-100 was added to the leading electrolyte (0.2%). The above electrolyte conditions were assessed by computer simulation.

The absolute mobility and the acid dissociation constants used in simulation are summarized in Table I. The pK_a value of TFA has not yet been reported. It was therefore assumed as 0.6 by analogy with trichloroacetic acid (pK_a = 0.64).

TABLE I

PHYSICOCHEMICAL CONSTANTS USED IN SIMULATION (25°C)

m_0 = absolute mobility ($cm^2\ V^{-1}\ sec^{-1}$) × 10^5.
pK_a = thermodynamic acidity constants, assumed values being used for Cl^- and trifluoroacetic acid.

Cations	m_0	pK_a	Anions	m_0	pK_a
β-Alanine	36.7*	3.552	Cl^-	79.08	−3
ε-Aminocaproic acid	28.8*	4.43	F^-	57.4	3.173
Creatinine	37.2*	4.828	Trifluoroacetic acid	42.7*	0.6
Histidine	29.6*	6 04	Pyruvic acid	42.3	2.490
Imidazole	29.5*	8.08	Phosphoric acid	35.1*	2.12
Tris(hydroxymethyl)aminomethane	29.5*	8.08		61.5*	7.470*
				71.5	12.360
Amediol	29.5*	8.78	Lactic acid	36.5	3.860
Ethanolamine	44.3	9.498			

*The absolute mobilities were obtained isotachophoretically and the other constants were taken from ref. 9.

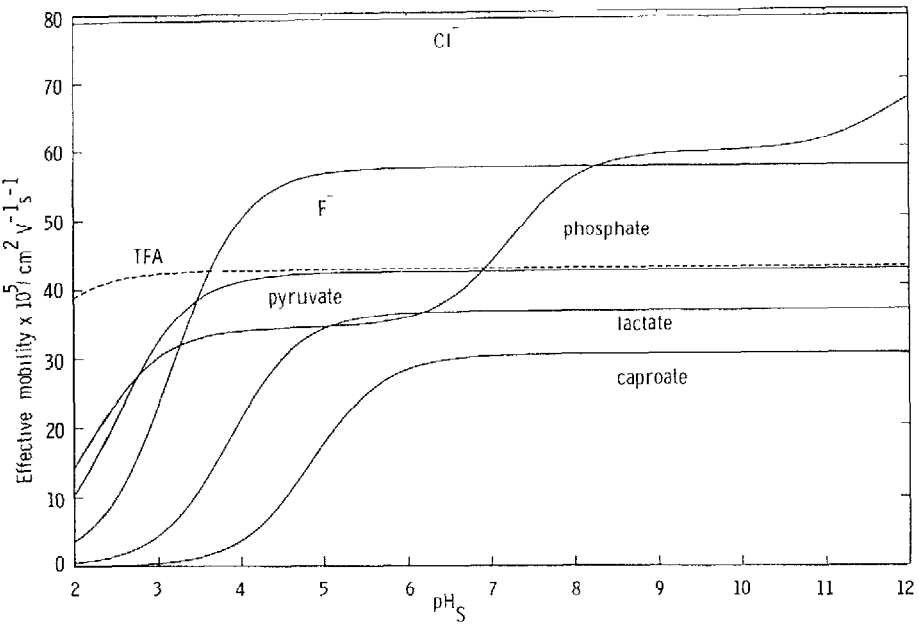


Fig. 1. The pH dependence of effective mobility of Cl^-, F^-, trifluoroacetate (TFA; broken curve), pyruvate, phosphate, lactate and caproate ions using absolute mobility and pK_a in Table I The curves are not for the isotachophoretically steady state.

Fig. 1 shows the pH dependence of effective mobilities of the treated samples using absolute mobility and thermodynamic pK_a values listed in Table I. Although the curves in Fig. 1 are not for the isotachophoretically steady state, the curves are useful for a rough estimation of the optimum pH range for the separation. As is apparent from Table I and Fig. 1, the absolute mobility of pyruvate and TFA ions differ slightly (4×10^{-6} cm^2 V^{-1} sec^{-1}); therefore, the separation of these ions may be impossible in the medium and high pH range at which these ions are in the fully charged state. On the other hand, the pK_a value of pyruvate is 2.49 and it differs from the pK_a of TFA by approximately 2; thus these acids may be separated in the low pH range utilizing the pH dependence of effective mobility. However, in the low pH range (< 4) the pH versus effective mobility curve of F^- intersects those of TFA and pyruvate, and the optimum pH may be very critical, supporting the reported narrow pH range for separation. For the precise estimation of separability of these ions, a simulation technique is necessary.

As shown in the previous paper, the qualitative index (R_E) at isotachophoretically steady state [7] can be simulated at any pH of the leading electrolyte (pH_L) and the separability of the samples can be estimated by comparing the simulated indices. The index, R_E, is the ratio of the potential gradient of a leading zone to that of a sample zone. The ratio is equal to the inverse relative mobility as follows:

$$R_E = E_S/E_L = h_S/h_L = \overline{m}_L/\overline{m}_S \quad (1)$$

where E, h and $\overline{m}$ denote potential gradients, step heights of isotachopherogram and effective mobilities, respectively, and L and S denote leading and

sample zones (ions). The difference of R_E values among samples is a measure of the separability at a certain pH_L, since the separability is closely related to the difference in the effective mobility of the objective samples. Practically, when the R_E values of two samples at the steady state differ by about 0.15, the samples may be separated.

The simulation conditions were as follows. The leading ion was 10 m*M* Cl^- and the detection of Br^- was not intended. Namely, the absolute mobility of Br^- (81.0×10^{-5} cm^2 V^{-1} sec^{-1}) is greater than that of Cl^-; therefore Br^- precedes Cl^-. For the terminating ion, caproate was used. Then, the pH values of the leading electrolytes (pH_L) were varied in the range 2.8—4.2 by β-alanine (Ala), 4.0—5.0 by ε-aminocaproic acid (AMC), 4.4—5.6 by creatine (Cre), 5.4—6.6 by histidine (His), 6.4—7.6 by imidazole (Im), 7.4—8.6 by tris-(hydroxymethyl)aminomethane (Tris), 8.4—9.2 by 2-amino-2-methyl-1,3-propanediol (amediol; Am), and 9.0—9.7 by ethanolamine (EA). In the above pH_L range, R_E values were simulated for lactic acid, phosphoric acid, pyruvic acid, TFA, and F^- at an isotachophoretically steady state. Fig. 2 shows the pH_L dependence on the R_E values. Apparently, in the high pH_L range (> approximately 4) the curves cross each other at several pH_L values, suggesting the difficulty of separation. In the low pH_L range the curve of F^- crosses those of pyruvate and TFA; however, they can be separated in the pH range approximately 3.4—3.6. Thus, the estimated optimum pH_L was in the

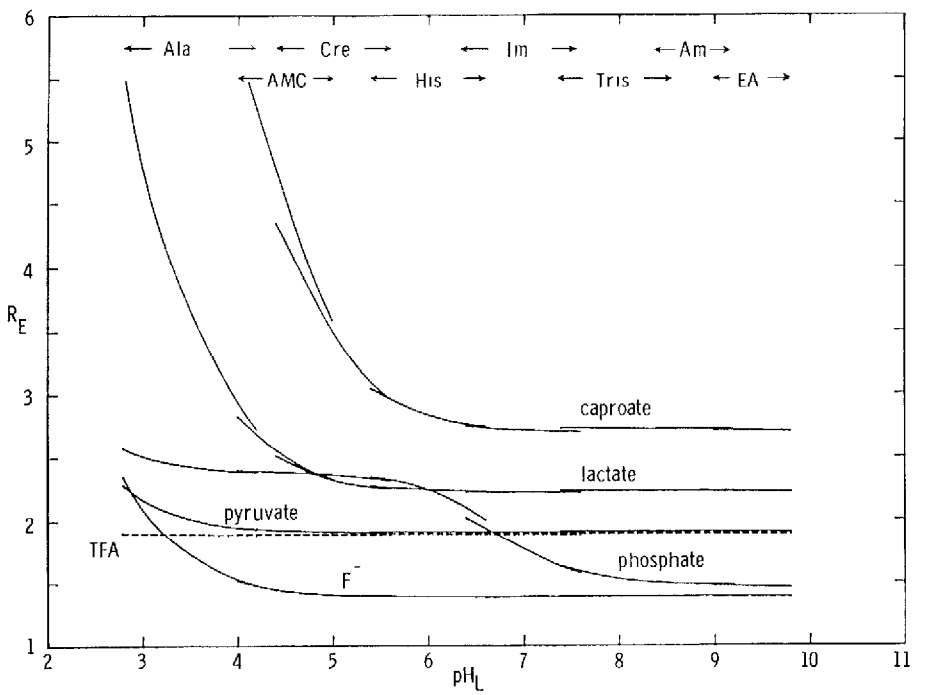


Fig. 2. The pH dependence of simulated R_E values of F^-, trifluoroacetic acid (TFA: broken curve), pyruvate, phosphate, lactate and caproate ions at isotachophoretically steady state. The leading electrolyte is 10 m*M* Cl^-. The buffers used were β-alanine (Ala; pH_L 2.8—4.2), ε-aminocaproic acid (AMC; 4—5), creatine (Cre; 4.4—5.6), histidine (His; 5.4—6.6), imidazole (Im; 6.4—7.6), tris(hydroxymethyl)aminomethane (Tris; 7.4—8.6), 2-amino-2-methyl-1,3-propanediol (amediol, Am; 8.4—9 2), and ethanolamine (EA; 9.0—9.7).

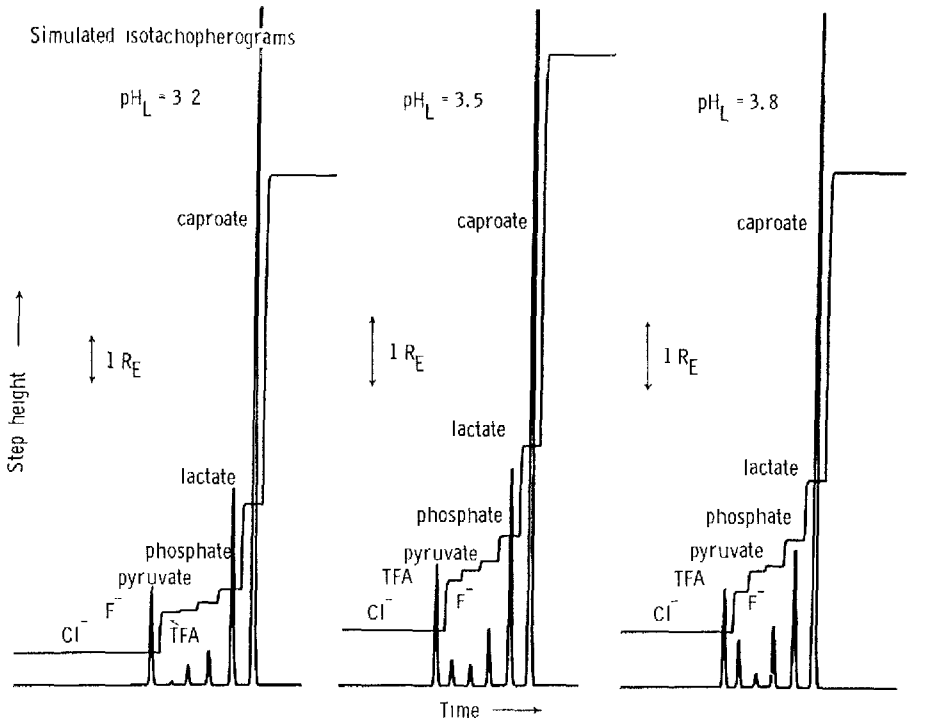


Fig. 3. The simulated isotachopherograms at pH$_L$ 3.2, 3 5, and 3.8 for equimolar F$^-$, TFA, pyruvate, phosphate, and lactate ions (buffer β-alanine). The leading ion is Cl$^-$ and the terminating ion is caproate.

very narrow pH$_L$ range and this estimation agreed with the experimentally assessed optimum pH$_L$ range.

Fig. 3 shows the simulated isotachopherograms at pH$_L$ 3.2, 3.5 and 3.8. At pH$_L$ = 3.2, F$^-$ (R_E = 1.92) and TFA (1.89) may form a mixed zone. At pH$_L$ 3.5, F$^-$ (R_E = 1.73), TFA (1.89) and pyruvate (2.02) can be separated, confirming the previous electrolyte conditions [3]. At pH$_L$ 3.8, the separation of TFA (1.89) and pyruvate (1.97) becomes difficult.

QUANTITATIVE ANALYSIS

A passing time of zone t (sec) of a sample n (nmol) can be expressed as

$$t = 10^{-6}\, n\, \kappa_S / I\, C_S^t\, \bar{m}_S \qquad (2)$$

where κ_S is the specific conductivity (S/cm), I the driving current (A), C_S^t the total concentration (M) of the sample in the separated zone, and $\bar{m}_S$ the effective mobility (cm^2 V^{-1} sec^{-1}) of the sample zone. The passing time can be calculated for a certain amount of sample using the simulated values of κ_S, C_S^t, and $\bar{m}_S$ under an arbitrary driving current I. Namely, a proportional coefficient between n (nmol) and t (sec) in the conventionally used calibration curve can be easily obtained by simulation technique. Fig. 4 shows the coefficients of the calibration curve (a) of treated samples under a 50 μA driving current in the

pH_L range of 3—10:

$$n = a\,t$$

$$a = C_S^t\,\bar{m}_S \times 50/\kappa_S \qquad (3)$$

Apparently from Fig. 4, the proportional coefficients depend not only on the mobility of the sample but also on that of the buffer ion used. The details of the quantitative aspects in isotachophoresis have been reported in Part I [8].

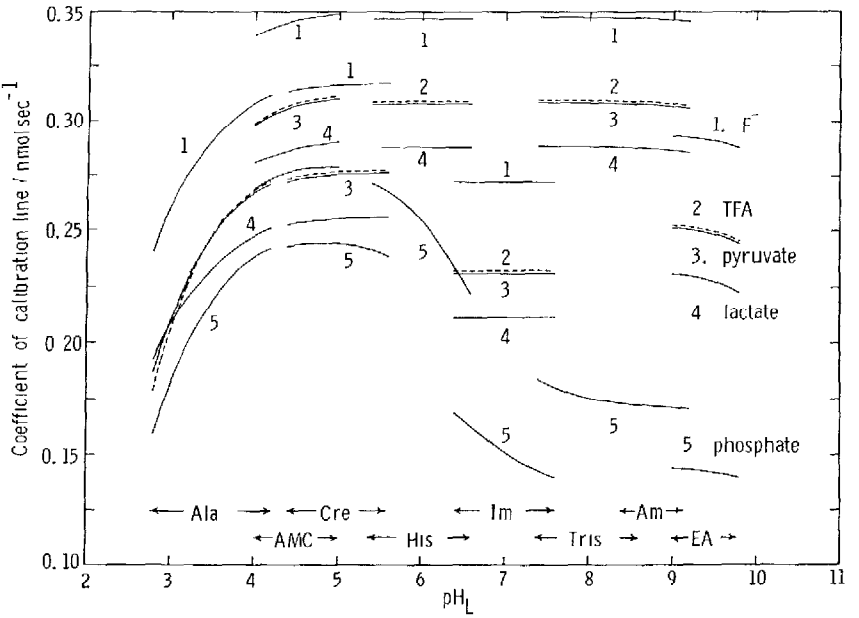


Fig 4. The simulated coefficient (a) of calibration line n (nmol) = $a\,t$ (sec) of F^-, trifluoroacetate (TFA; broken curve), pyruvate, phosphate, and lactate ions. For the electrolyte system used, see legend of Fig. 2.

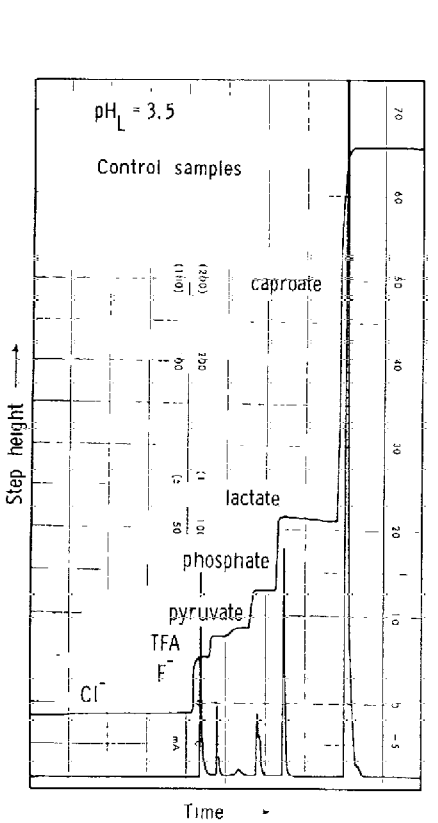
EXPERIMENTAL

To identify the separated TFA, a mass spectrogram of a prepared target fraction was obtained. For this purpose the optimized electrolyte system was used. The leading electrolyte was 10 mM hydrochloric acid and the pH_L was adjusted by β-alanine at 3.5. The terminating electrolyte was 10 mM caproic acid. The pH measurements were carried out using an Iwaki glass pH meter Model 225, and the isotachopherograms were obtained using a Shimadzu isotachophoretic analyser, IP-2A, equipped with a potential gradient detector designed for preparative use. The temperature of the separation compartment was thermostatted at 25°C. A main separating tube, 6 cm × 0.5 mm I.D., was connected to a preseparating column, 2 cm × 1 mm I.D. The driving current was stabilized at 50 μA during the detection of sample zones. To measure R_E values precisely, an asymmetric potential of the potential gradient detector was corrected using the simulated R_E values of an internal standard, lactic acid. The reagents used for control samples of the urinary acids and the metabolites

were commercial guaranteed grade. An isotachopherogram of urine from a patient anaesthetized by halothane was also obtained without any pretreatment. A mass spectrogram of prepared TFA was obtained using an Hitachi mass spectrometer Model RMS-4. The experimental conditions were: chamber voltage, 80 V; total emission, 80 μA; target current, 50 μA; and chamber temperature, 250°C.

RESULTS AND DISCUSSION

Fig. 5 shows an isotachopherogram of control samples, lactic acid, phosphoric acid, pyruvic acid, fluoride, and TFA at pH_L 3.5. As expected the mixture was separated under the electrolyte conditions. A good agreement was obtained between the simulated isotachopherogram in Fig. 3 and the observed one in Fig. 5, confirming the present simulation. Table II shows the observed and simulated R_E values of these acids, their effective mobility and concentrations in the separated zones. Except for F^- the observed and the simulated R_E values agreed well. Then, these electrolyte conditions were applied to urine samples. Fig. 6 shows the isotachopherogram of urine of a patient who was



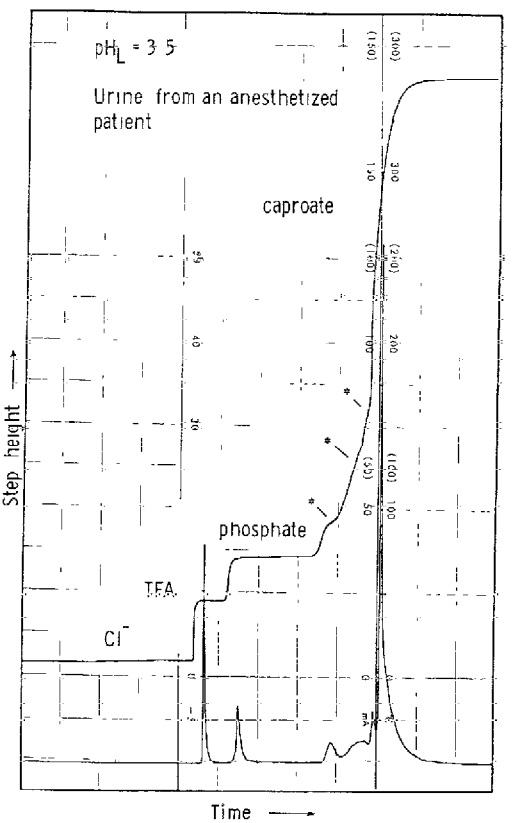


Fig. 5. The observed isotachopherograms of control samples: F^-, TFA, pyruvate, phosphate, lactate ions at pH_L 3.5 under 50 μA driving current.

Fig. 6. The observed isotachopherograms of urine from an anaesthetized patient at pH_L 3.5 under a driving current of 50 μA. Asterisks show unidentified components.

TABLE II

OBSERVED AND SIMULATED R_E VALUES OF FLUORIDE, TRIFLUOROACETATE, PYRUVATE, PHOSPHATE AND LACTATE, EFFECTIVE MOBILITY AND CONCENTRATION OF ZONE CONSTITUENTS AT pH_L 3.5 (25°C)

Leading electrolyte = 10 mM Cl^-, β-alanine.
R_E = ratio of potential gradients, E_S/E_L.
$\overline{m}_S$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of sample ion × 10^5.
pH_S = pH of sample zone.
C^t_S = total concentration (mM) of sample.
$C^t_{B,S}$ = total concentration (mM) of buffer ion.
$\overline{m}_{B,S}$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of buffer ion × 10^5.
κ_S = specific conductivity (mS/cm) of sample zone.
I = ionic strength × 10^3.

Ions	B_E		$\overline{m}_S$	pH_S	C^t_S	C^t_B	$\overline{m}_{B,S}$	κ_S	I
	Corr.*	Calc**							
Fluoride	1.62	1.73	43 00	3.729	8.887	16.35	−14.19	0.656	7.07
TFA	1.81	1.89	39.44	3.642	7.539	15.52	−15.82	0.601	7.53
Pyruvate	1.98	2 02	36.99	3.683	7.531	15.45	−15.05	0.564	7.11
Phosphate	2.42	2.37	31.47	3.711	6 746	14.87	−14.53	0.480	6.59
Lactate	3.67	(std.)	20.30	3.996	7 083	14.78	−9.547	0.310	4.21

*Corrected R_E values, the internal standard was lactate ion.
**Simulated R_E values.

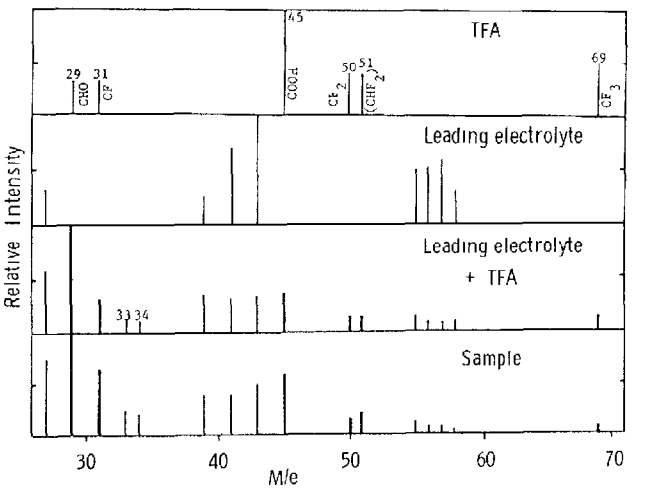


Fig. 7. The observed mass spectra of neat trifluoroacetic acid (TFA), leading electrolyte (pH_L 3.5, β-alanine buffer), leading electrolyte + TFA, and prepared TFA zone.

anaesthetized by halothane. A zone corresponding to TFA could be found as well as phosphoric acid, lactic acid, and unidentified anions, although F^- and pyruvate could not be detected in this case. This assignment of zones was confirmed by mass spectometry. Fig. 7 shows the mass spectra obtained of neat TFA, leading electrolyte, leading electrolyte + TFA, and the separated zone of

TFA in urine from a patient anaesthetized by halothane. The spectrum of the separated fraction has the following fragment ion peaks, m/e: 29 (CHO), 31 (CH), 45 (COOH), 50 (CF_2), 51 (CHF_2), 69 (CF_3), which can be assigned to the fragments of TFA in comparison with those of neat TFA. The molecular ion peak of m/e = 114 was not detected. The other peaks can be assigned for the fragments of β-alanine in leading and TFA zones.

The coefficient of the calibration curve of TFA was obtained under a 50 μA driving current using the above electrolyte system. The experimentally obtained relation between sample amount n (nmol) and zone-passing time t (sec) was as follows:

$$n = 0.25t \quad (4)$$

The coefficient of correlation of the obtained slope was 0.997 for fifteen replicates, varying n in the range 1.5—15 nmol. The simulated coefficient was 0.247 (nmol/sec) and the agreement was good. The minimum detectable amount of TFA was 1.5 nmol and it took 6 sec for detection.

Thus the isotachophoretic technique was demonstrated to be useful in analysis of urinary TFA without time-consuming pretreatment. This is quite important in clinical analysis. And the use of a computer simulation technique has proved to be useful for the separation and quantification problem in isotachophoresis.

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