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Note

Some quantitative aspects of UV detection in capillary isotachophoresis as applied to bioavailability studies

M. SVOBODA* and X. SVOBODOVÁ

Výskumný ústav liečiv (Drug Research Institute), CS-900 01 Modra (Czechoslovakia)

and

J. VACÍK

Faculty of Science, Charles University, Albertov 2030, CS-120 00 Prague 2 (Czechoslovakia)

The analysis of drugs in biological fluids for the purpose of bioavailability assessment requires sensitive and selective methods for the detection of the compound of interest. Analytical isotachophoresis combined with UV detection may be one of the methods of choice. The objective of this study is to propose a new reliable means of quantitation useful in conjunction with absorptiometric detection in analytical isotachophoresis and to demonstrate its applicability to the determination of trace amounts of biologically active compounds in complex matrices.

The concept of zone area as an integrated zone absorbance useful in zone electrophoresis or elution chromatography cannot be directly transferred to isotachophoresis because of the rather different zone shapes in these techniques [1]. In isotachophoresis the concentration profile of the zone along the longitudinal axis of the separation compartment is almost rectangular even for very short zones [1].

THEORY

If a sufficiently small amount of UV-absorbing substance is introduced as a sample, it may produce a zone shorter than the UV detector aperture width, but still possessing quite a rectangular concentration profile [1, 2]. Indeed, this is a very typical situation in isotachophoresis. As first noticed by Arlinger [3], the step height of such a short zone is no longer constant, but it becomes

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dependent on the physical zone length or on the amount of the compound injected. Partial analysis of the step height behaviour, given by Svoboda and Vacík [2], has revealed that for such short zones the step height expressed in terms of absorbance D

$$D = 1 - T \tag{1}$$

(T denoting transmittance) becomes a linear function of the amount injected and may thus be used for quantitation. The apparent zone length (read as the zone width in its half height) becomes independent on the amount injected and becomes equal to the detector aperture width:

$$D_{peak} = D_0 \cdot L/d \tag{2a}$$

$$w = d = \text{constant} \tag{2b}$$

The various interrelations between physical zone length L, maximum zone absorbance D_{peak}, apparent zone length w, and integrated absorbance are summarized in Fig. 1 together with the schematic appearance of the zone at various physical zone lengths. In both eqn. 2a and Fig. 1, d denotes the detector aperture width. Using the model of rectangular detector aperture and planar zone boundaries, the straightforward calculation of integrated zone absorbance is easily done, yielding the following expressions relating the physical zone length and integrated absorbance S (zone area), respectively

$$S = D_0 \cdot L \tag{3a}$$

$$S = D_0 \cdot L + D_1 \cdot d/2 \tag{3b}$$

where D_0 denotes the correct response [3] of the compound of interest and D_1 the correct response of the zone following or preceding the zone of interest.

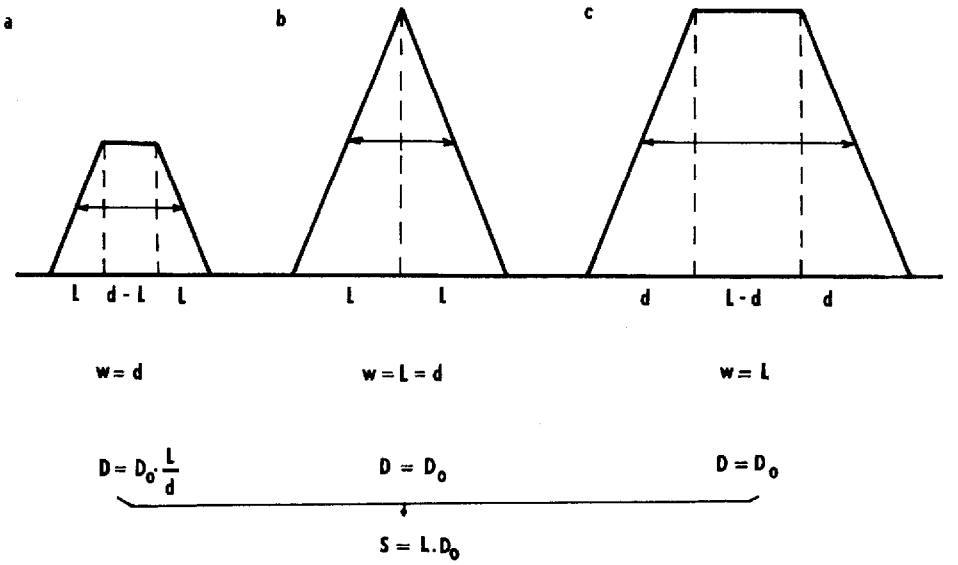

Fig. 1. Interrelations between various zone parameters: (a) zone length shorter than detector aperture width; (b) zone length equal to the aperture width; (c) zone length greater than the aperture width. For explanation of symbols see text.

Eqn. 3a holds for a single UV-absorbing zone passing the detector aperture, eqn. 3b holds for a strongly absorbing short zone followed or preceded by a less absorbing long zone of compound 1 (possibly internal standard).

More involved models including circular detector aperture and paraboloidal zone boundaries were analyzed numerically to verify the usefulness of zone area as a means of quantitation in more real circumstances [4]. The results of these simulations are exemplified in Table I, where slopes and intercepts of regression lines relating the calculated zone area and physical zone length

$$S = a \cdot L + b \tag{4}$$

are given. In each case the model yielded linear dependence with insignificant intercept and slope close to correct response (cf. eqn. 3a).

TABLE I

ZONE AREA AS A FUNCTION OF ITS PHYSICAL LENGTH

$S = a \cdot L + b$. Single absorbing zone passing through the detector.

Aperture	Zone boundary	a	s_a*	$10^2\ b$	$10^2\ s_b$*
Circular	Planar	8.444	0.006	0.4	1.1
Circular	Paraboloidal	8.41	0.04	3	6
Rectangular	Paraboloidal	8.06	0.03	2	5

*Standard error of the estimates of a and b (as calculated from the Monte Carlo simulation [4]).

EXPERIMENTAL

The compound under study [α-arylalkylamino-ω(N_q-aryl-1,q-diaza(substituted)cycloalk-1-yl) alcohol] is denoted here as VULM 120; it was synthesized in the Drug Research Institute (Modra, Czechoslovakia). It possesses three tertiary amine functions, two of them being dialkylarylamine groups, the third being of the trialkylamine type. Its molecule carries two relatively weak isolated aromatic chromophores and its net charge in the vicinity of pH 5 is about 1.5 electron charges. Considering the modest absorptivity of the compound under study, a low absorbing internal standard (tetraphenylarsonium ion) was chosen. A detailed scheme of sample preparation prior to isotachophoresis is shown in Fig. 2.

Isotachophoresis was done using the instrument constructed in the Drug Research Institute, Modra, equipped with a 28 cm × 0.5 mm I.D. FEP capillary (Kablo Vrchlabí, Czechoslovakia) and filter photometric UV detector. A high-pressure mercury arc lamp (HQE 40; Narva, G.D.R.) and 254-nm interference filter (UV KIF 254; C. Zeiss, Jena, G.D.R.) were used. The leading electrolyte consisted of sodium acetate brought to pH 5 with acetic acid and diluted to give a final Na^+ concentration of 2 mmoles/l. After dilution, the pH was checked and readjusted to 5 ± 0.05 by addition of either sodium acetate (2 mmoles/l solution) or a few drops of concentrated acetic acid. No additives to this electrolyte were used. The terminating electrolyte used was ca. 0.2 mole/l

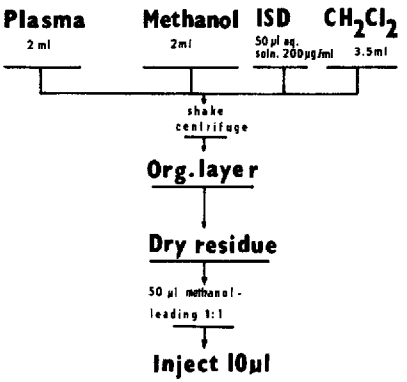

Fig. 2. Scheme of sample preparation prior to isotachophoresis. The dichloromethane extract was reduced to dryness on a water-bath at 55—65° C. ISD = internal standard (200 µg/ml aqueous solution of tetraphenylarsonium chloride).

glycine. All chemicals used were p.a. grade preparations (Lachema, Brno, Czechoslovakia) except for tetraphenylarsonium chloride (p.a.; Fluka, Buchs, Switzerland) and dichloromethane (Merck, Darmstadt, G.F.R.; Uvasol grade). Typical recordings of sample and blank runs are shown in Fig. 3. In both cases 2 ml of either control or spiked plasma were subjected to the sample preparation procedure given in Fig. 2. and 10 µl of the resulting solution were analyzed.

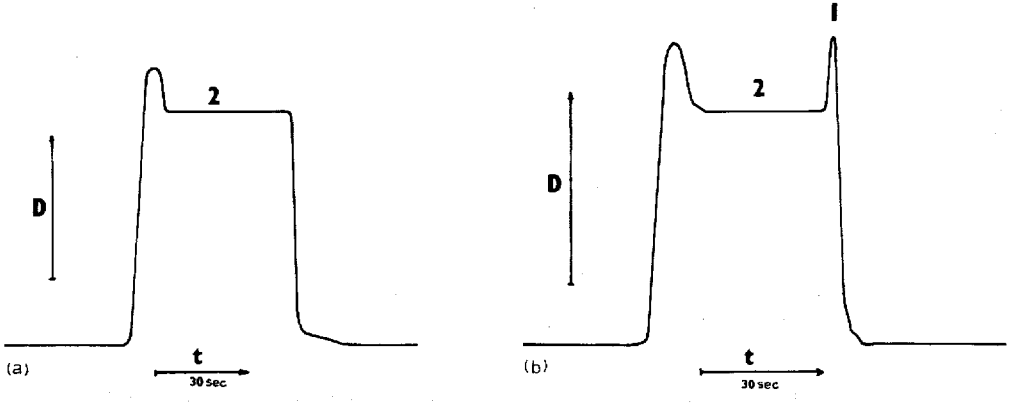

Fig. 3. Isotachopherograms of (a) typical blank and (b) sample runs. 1 = VULM 120, 2 = internal standard; driving current 20 µA. In both cases 2 ml of plasma were extracted.

The results were quantitated using the internal standard method in terms of the zone area ratios

$$c_{\text{plasma}} = a(S_{120}/S_{\text{TPA}}) + b \qquad (5)$$

where c_{plasma} is the unknown plasma concentration, S_{120} and S_{TPA} are the zone areas of VULM 120 and tetraphenylarsonium cation, respectively. The standard curve, obtained by analyzing rat plasma samples spiked with known amounts of VULM 120, is shown in Fig. 4. The curve is linear from the detection limit up to the highest concentration tested (6 µg/ml plasma). The detection limit was

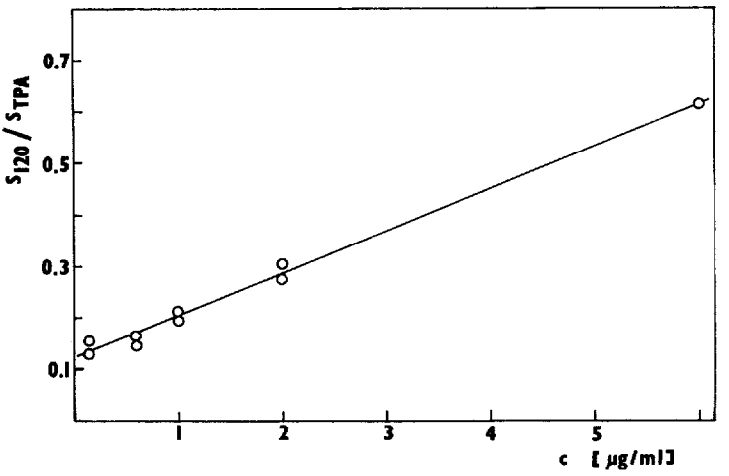

Fig. 4. Standard curve obtained by analyzing plasma samples spiked with known amounts of VULM 120. c denotes concentration of VULM 120 in plasma; S_{120}/S_{TPA} = zone area ratio (see text).

TABLE II

SOME REPRESENTATIVE RESULTS OF PLASMA CONCENTRATION OF VULM 120

c_{plasma} = (12.4 ± 0.2) · (S_{120}/S_{TPA}) − (1.54 ± 0.02); n = 9. All concentration data are in µg/ml.

Concentration expected (c_0)	Concentration found (c)	σ*	σ_e**	n***
0.40	0.39	0.04	0.052	6
0.50	0.53	0.07	0.053	3
1.00	1.03	0.04	0.061	3

*Standard error estimated from the spread of the measured data.
**Standard error estimated from the calibration curve.
***Number of measurements.

estimated from the spread of the standard curve points and it is about 80 ng/ml plasma. The slope and intercept of the standard curve together with their standard errors and some representative data are given in Table II.

DISCUSSION

Based on the reported results, the zone area (in terms of integrated zone absorbance) may be considered a very useful means of quantitation in capillary isotachophoresis, improving the sensitivity and accuracy even in such highly demanding tasks as analysis of trace amounts of biologically active compounds in the complex matrices of biological fluids. Even though the UV-spike method based on step height measurement gave rise to some analytical methodologies with detection limits in the low picomole range (see, for example, refs. 2 and

5), it suffers from serious disadvantages. The linear relation may be affected by the non-rectangular shape of detector aperture. Moreover, both the UV-spike and classical zone-length methods become very inaccurate if the physical zone length approaches the detector aperture width. The use of zone area, which is a monotonic function (linear in most cases) of the physical zone length (or the amount injected) regardless of the physical zone length, may help to overcome these difficulties.

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