VINYL CHLORIDE / MEDICAL Rec'd 11-26-84 SCSD

[4] Görg, A., Weser, J., Westermeier, R., Postel, W., Weidinger, S., Patutschnick, W. and Cleve, H., *Hum. Genet.* 1983, *64*, 222–226.
[5] Arnaud, P. and Allen, R. C., in: Allen, R. C. and Arnaud, P. (Eds.), *Electrophoresis '81*, Walter de Gruyter, Berlin 1981, pp. 495–504.
[6] Fagerhol, M. K. and Cox, D. W., in: Harris, H. and Hirschhorn, K. (Eds.), *Adv. Human Genet.* 1981, *11*, 1–62.
[7] Constans, J., Viau, M. and Gouaillard, C., *Hum. Genet.* 1980, *55*, 119–121.
[8] Weidinger, S., Cleve, H. and Patutschnick, W., *Z. Rechtsmed.* 1982, *88*, 203–211.
[9] Talamo, R. C., Langley, C. E., Reed, C. E. and Makino, S., *Science* 1973, *181*, 70–71.
[10] Görg, A., Postel, W., Westermeier, R., Gianazza, E. and Righetti, P. G., *J. Biochem. Biophys. Methods* 1980, *3*, 273–284.
[11] Görg, A., Postel, W., Westermeier, R., Righetti, P. G. and Ek, K., LKB Application Note Nr. 320, 1982.
[12] Ritchie, R. F. and Smith, R., *Clin. Chem.* 1976, *22*, 497–499.
[13] Weidinger, S., Schwarzfischer, F. and Cleve, H., *Proceedings of the 10th International Congress of the Society for Forensic Haemogenetics*, München 1983, pp. 387–392.

REV 8(E)

CUSTOMER'S COPY
COMPLIES WITH COPYRIGHT LAW
CHEMICAL ABSTRACTS SERVICE

Ludmila Křivánková[1]
Eva Samcová[2]
Petr Boček[1]

[1]Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Brno
[2]Department of Medical Chemistry and Biochemistry, Medical Faculty of Hygiene, Charles University, Prague

(82) (84) (14) (92) CS (94) 611 42

Determination of thiodiacetic acid in urine of people exposed to vinyl chloride by analytical capillary isotachophoresis

A method is introduced for the determination of thiodiacetic acid in urine by analytical capillary isotachophoresis with column coupling. Thiodiacetic acid is one of the final metabolites of carcinogenic vinyl chloride and appears at increased levels in the urine of people exposed to vinyl chloride vapours. The method enables the direct analysis of samples of 10 µl of urine without any pretreatment in about 45 min. The lowest detectable concentration of thiodiacetic acid was about 6×10^{-6} mol/liter and the reproducibility of the analyses in the range of $2-15 \times 10^{-5}$ mol. per liter was about 3 rel. %.

Introduction

The oncogenic properties of vinyl chloride (VC), the monomer of polyvinyl chloride (PVC), which is one of the most widely used plastics, have been known since 1971 [1]. Thus, exposure to VC vapours must be monitored above all in the production of PVC, where the workers are open to the highest risk. The first step of VC metabolism in the organism is the oxidation by microsomal monooxygenase to chlorethylenoxide, which is then responsible for most of the deleterious effects of VC. One of the final metabolites of VC in the organism is thiodiacetic acid (TDA). The direct correlation between the exposure of the organism to VC and the excretion of TDA in urine has already been proved [2, 3]. Small amounts of TDA can even be present in the urine of persons not exposed to VC vapours, reflecting the diet of the person. However, for persons working in PVC production, the content of TDA in urine increases considerably. Hence, the content of TDA in urine of these workers can be a criterion for health protection in the respective factories. At present TDA in urine is determined by gas chromatography after its preliminary extraction and esterification [2–4]. This multi-stage procedure is laborious, time-consuming and is often subject to considerable errors. Therefore, it was desirable to work out a fast and reliable method for the direct determination of TDA in the unprocessed urine.

Isotachophoresis (ITP) has already proved to be an expedient analytical method for environmental control and clinical analyses in cases of occupational hazard. Sollenberg and Baldesten [5], Vesterberg and Sollenberg [6], and Zschiesche *et al.* [7] analysed the urine of people exposed to the vapours of styrene, toluene and xylene and determined successfully the metabolites, *i. e.*, mandelic, phenylglyoxylic, hippuric and methylhippuric acids. The concentrations of these metabolites in the urine samples were in the order of 10^{-4} mol/liter and the detection limit of the method was in the order of 10^{-5} mol/liter. Øvrebø *et al.* [8] analysed formic acid in the plasma of people intoxicated with methanol where the concentration level of formic acid was in the order of 10^{-4} mol/liter. These works encouraged us to apply ITP to our problem and we have worked out a method which enables the determination of concentrations of TDA in a sample of 1–10 µl of urine during 45 min without any pretreatment of the sample.

2 Materials and methods

2.1 Instrumentation

For ITP the CS Isotachophoretic Analyser, Spišská Nová Ves, Czechoslovakia, was used. This instrument is equipped with a column coupling system consisting of two polytetrafluoroethylene (PTFE) capillaries of 0.8 and 0.3 mm I. D. serving the pre-separation and analytical runs, respectively. Each capillary is equipped with the conductivity detector. The bifurcation block between the two capillaries mentioned above is equipped with a side branch leading to the auxiliary electrode and enables us to select the interesting part of sample

Correspondence: Dr. Petr Boček, Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Leninova 82, CS-611 42 Brno, Czechoslovakia

Abbreviations: VC: Vinyl chloride; PVC: Polyvinyl chloride; TDA: Thiodiacetic acid; ITP: Isotachophoresis; LE: Leading electrolyte; TE: Terminating electrolyte

© Verlag Chemie GmbH, D-6940 Weinheim, 1984

0173-0835/84/0408-0226$2.50/0

R&S 005194

R&S 005195

zones (heart-cutting) for further analysis. The migration path between the injection port and the tell-tale detector of the pre-separation capillary is about 19 cm, the migration path between this detector and the bifurcation point is 5 cm. The length of the analytical capillary is 17 cm. Analyses were carried out at room temperature. The stabilized driving currents were 200 μA and 45 μA in the pre-separation and analytical runs, respectively. The time of the analysis in question was dependent on the sample composition and, on average, was about 45 min.

2.2 Electrolytes and chemicals

The solutions of 10 mmol/liter adjusted with β-alanine to the desired values of pH were used as the leading electrolytes (LEs) in the pre-separation and analytical capillaries. The solutions contained hydroxypropyl-cellulose of 0.2 % w/v. The terminating electrolyte (TE) was 10 mmol/liter acetic acid in all cases. The HCl and acetic acid used were of analytical grade (Lachema, Brno, Czechoslovakia); β-alanine, pure, was from Loba-Chemie, Vienna, Austria; hydroxypropyl-cellulose was from Ega-Chemie, Albuch, Federal Republic of Germany. Thiodiacetic (thiodiglycolic) acid, *p. a.*, was from Fluka A. G., Federal Republic of Germany. The other acids used to identify the zones were of analytical grade (Lachema, Brno). All chemicals were dissolved in distilled water deionized with mixed-bed ion-exchangers Ostion AD ans KS (Spolchemie, Usti n. L., Czechoslovakia).

2.3 Urine samples

There was no pretreatment of urine samples. Samples were stored at 0–4 °C for 7–10 days and no changes in the concentration of thiodiacetic acid were observed during this period.

3 Results and discussion

3.1 Interference from bulk anionic components

TDA is a bivalent acid with pK values 3.15 and 4.13 [8] in water and can be easily analysed in the whole pH range practically used in anionic ITP. The concentrations of TDA in urine in the order of $1.5–15 \times 10^{-5}$ mol/liter also represent no problem for common isotachophoretic instrumentation equipped with conductivity, gradient or UV absorption detection. However, the analytical problem stems from the sample character. Urine contains on average about 8 g of chlorides, 23 g of urea, 2.1 g of inorganic phosphates, 1.2 g of creatinine, 0.7 g of uric acid, 0.08–0.8 g of hippuric acid [9], 0.05–0.46 g of citric acid [10] *etc.* in one liter, which corresponds to the concentrations in mol/liter of 0.23, 0.38, 0.015, 0.01, 3.47×10^{-3}, $0.465–4.65 \times 10^{-3}$, and $0.275–2.4 \times 10^{-3}$, respectively. Evidently, the concentrations of the bulk anionic components are several orders of magnitude higher than the average concentration of TDA and may strongly interfere with its determination. This was the reason why the working procedure and instrumentation employing the "column coupling technique" [11] was used. The goal of this technique is that good pre-separation of the interfering bulk components from the analysed species is achieved in the pre-separation run, and, further, a narrow region of the substances to be analysed is then quantitatively trapped into the analytical capillary when passing the bifurcation block. Subsequently, the complete separation and the quantitative detection is achieved in the analytical run. This procedure is analogous to the heart-cutting technique commonly known in chromatography [12]. The range where the suitable electrolyte systems were looked for was restricted to the leading electrolyte of pH 3–5 with chloride as the leading ion and acetate as the terminating ion. Anions of acids weaker than acetate were expected to migrate in the elution way in the terminator and thus to be eliminated from the system of isotachophoretic zones.

3.2 Separations at different pH of the leading electrolyte

The sequence of zones of important substances in the range mentioned above follows from Fig. 1 where the relative step-height (the step-height represents the reciprocal conductivity) *vs.* pH of the LE is plotted. It is obvious from the figure that for pH 3.5–3.7 the zone of TDA migrates together with the phosphate zone and, moreover, at pH 3.9–4.1 the citrate zone joins them. Hence, the suitable pH range of the LE in the pre-separation and analytical capillaries seemed to be higher than 4.0 or lower than 3.5. We checked both alternatives.

In Fig. 2, there are records of separations carried out at the higher pH. In accordance with Fig. 1, the zone of TDA migrated before citrate and phosphate together with other more mobile acids (*e. g.* fumaric acid). For the zones between chlorides and citrates (together with phosphates) the heart-cut was applied and they were further analysed during the analytical run in the analytical capillary. For the identification and the quantitative assay of the TDA zone, the standard addition technique was used (see Fig. 2c). It is obvious that this procedure gave good separation in the analytical capillary. However, the sensitivity of analyses was too low due to the low separation capacity of the pre-separation capillary. It was tested experimentally that the separation capacity of the isotachophoretic system used was sufficient in this case to separate a maximum of 3 μl of urine. With higher volumes of injected urine, mixed zones of TDA and citrate were formed in the pre-separation capillary. For samples larger than 3 μl, the dependence of the step-length of TDA on the volume of injected urine enriched with TDA was not linear.

Therefore, the lower pH range was tested where phosphate migrates in front of TDA and may positively affect the separa-

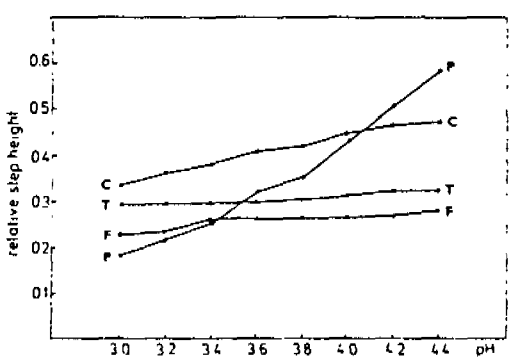

Figure 1. The dependences of the relative step-height *vs.* pH of the LE of some acids present in urine. LE: 10 mmol/liter HCl + β-alanine + 0.2 % w/v hydroxypropyl cellulose. TE: 10 mmol/liter acetic acid. P – phosphates, C – citrates, T – thiodiacetates, F – fumarates.

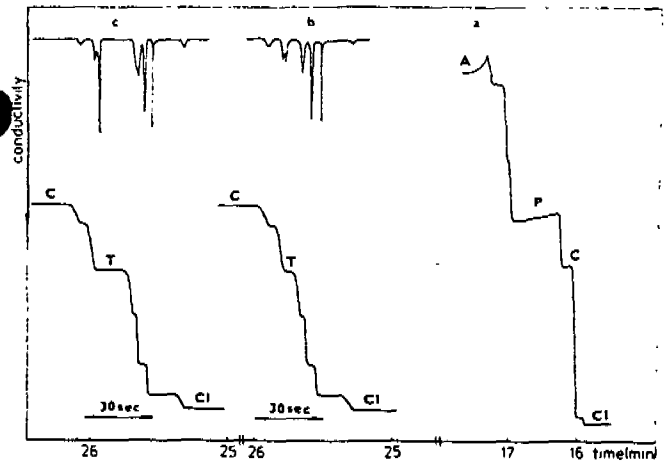

Figure 2. Isotachophoretic analysis of 2 µl of normal urine. LE in both capillaries: 10 mmol/liter HCl + β-alanine, pH 4.4, 0,2 % w/v hydroxypropyl cellulose. TE: 10 mmol/liter acetic acid. (a) The record of the pre-separation run. Driving current 250 µA, chart speed 0.5 mm/s. Cl – chlorides, C – citrates, P – phosphates, A – acetates. (b) The record of the analytical run. Driving current 45 µA, chart speed 1 mm/s. Cl – chlorides, T – thiodiacetates, C – citrates. (c) as in (b), 1 nmole of thiodiacetic acid added. Cl – chlorides, T – thiodiacetates, C – citrates. In the upper part of (b) and (c), the derivative of the detector signal is recorded.

tion capacity for the substances migrating behind it [13]. In the range of pH lower than 3.4, in agreement with the results of Fig. 1, the zone of TDA migrated between phosphate and citrate zones. The part of sample zones between phosphates and citrates was then trapped into the analytical capillary and analysed subsequently. Good separation was reached (see Fig. 3). The positive effect of the zone of phosphate upon the separation capacity for TDA and substances migrating in its neighbourhood was tested experimentally and it was found that volumes up to 10 µl of untreated urine can be successfully analysed. The calibration curve was constructed by employing the standard addition technique and the plot of the step length of TDA *vs.* its amount added to the normal urine was linear in the range 0–3 nmoles. Hence, the LE of pH 3.4 seemed to be suitable for the analyses of TDA in urine. However, during practical analyses of the urine of people exposed to VC, another problem appeared. In these samples, some other substances were found, the concentrations and effective mobilities of which were close to those of TDA. It is probable that these substances are chloroderivatives of carboxylic acids. They interfered with the analysis to such an extent that it was necessary to perform the comparison with further analysis of the sample enriched with a known amount of TDA to identify reliably the zone of TDA. Thus, the time of analysis was prolonged to 2 × 45 min. To solve this complicated situation we tried to increase the selectivity of the separation by using the combination of LEs in such a way that LE of pH 3.4 was in the pre-separation capillary and LE of pH 4.3 was in the analytical capillary. As the heart-cut for the analytical run only the zones of acids between phosphate and citrate were trapped into the analytical capillary. The regions of acids migrating in front of the heart-cut and behind it were eliminated from the separation capillary system by their migration to the auxiliary electrode (side branch of the bifurcation block). Using such a procedure, TDA migrated in the analytical capillary in front of citrate together with a small number of well separated zones of other acids and could be easily and unambiguously identified according to its step height in the record. The combination of LE of pH = 3.4 and

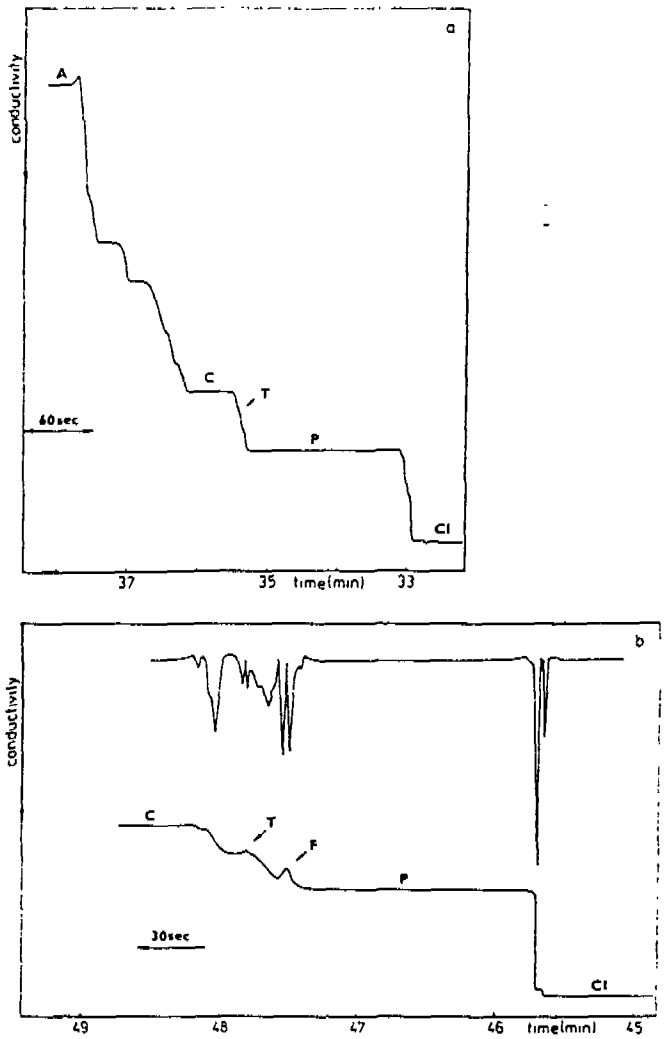

Figure 3. Isotachophoretic analysis of 5 µl of normal urine containing 1.98 mmol/liter thiodiacetic acid added. LE: 10 mmol/liter HCl + β-alanine, pH 3.3 + 0.2 % w/v hydroxypropyl cellulose in both capillaries. TE: 10 mmol/liter acetic acid. (a) The record of the pre-separation run. Driving current 200 µA, chart speed 0.5 mm/s. Cl – chlorides, P – phophates, T – thiodiacetates, C – citrates, A – acetates. (b) The record of the analytical run where the heart-cut of the sample zones between phosphates and citrates was analysed. Driving current 40 µA, chart speed 1 mm/s. Cl – chlorides, P – phosphates, F – fumarates, T – thiodiacetates, C – citrates. In the upper part of the figure the derivative of the detector signal is recorded.

LE of pH = 4.3 in the pre-separation and analytical capillaries, respectively, is advantageous and solves the problem of interfering acids (Fig. 4).

3.3 Calibration curve and reproducibility

The calibration curve (step length in the record *vs.* the amount of TDA added to urine) was linear in the concentration range in question and passed through zero (see Fig. 5; the amount of TDA originally present in urine was subtracted). The dependence of the step length of thiodiacetic acid on the volume of the analysed urine was checked up to the injection of 10 µl and was linear. Hence, the procedure proposed can be satisfactorily used for the qualitative and quantitative determination of TDA in urine of persons both exposed and not exposed to VC. Considering that the increasing volume of injected urine considerably prolongs the time needed for the analysis due to the

R&S 005196

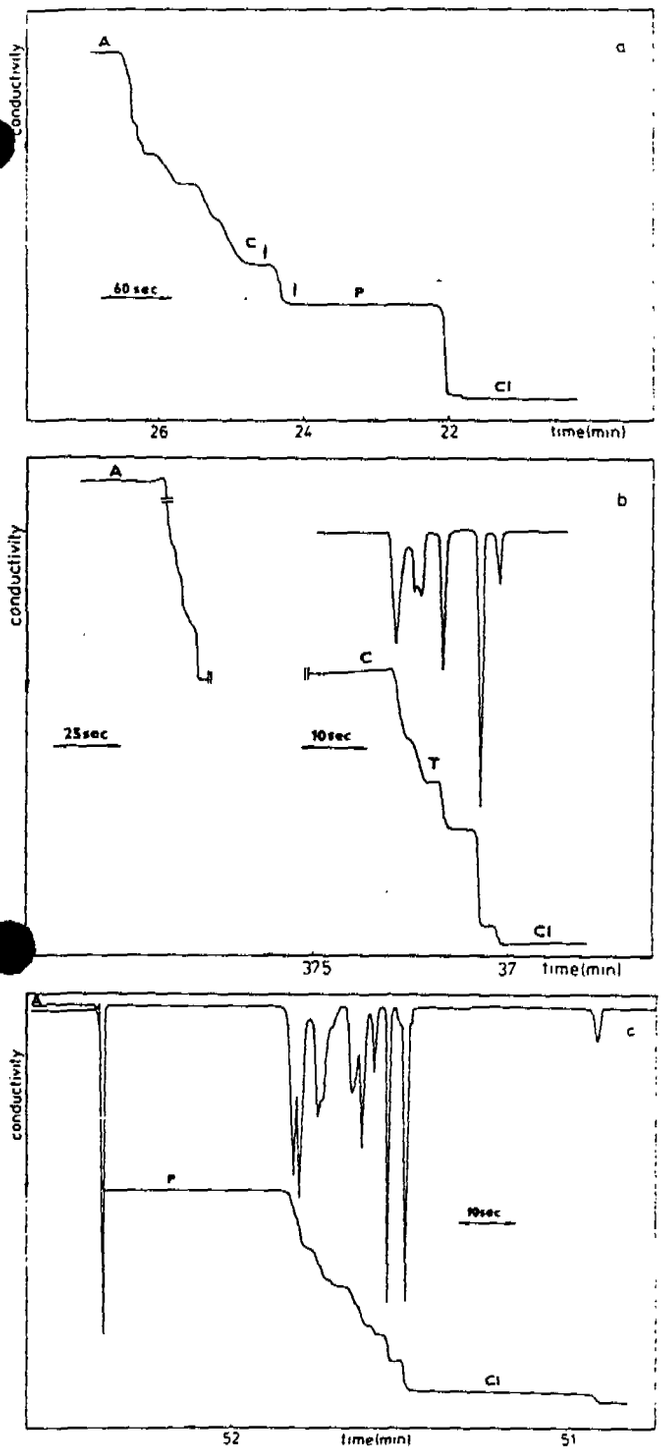

Figure 4. Analysis of 5 µl of urine of a person exposed to vinyl chloride by employing LE of different pH in the pre-separation and analytical capillaries. (a) The record of the pre-separation run. LE: 10 mmol/liter HCl + β-alanine, pH 3.4, + 0.2 % w/v hydroxypropyl cellulose. TE: 10 mmol/liter acetic acid. Driving current 200 µA, chart speed 0.5 mm/s. Cl – chlorides. P – phosphates. C – citrates. A – acetates. The section between arrows indicates the heart-cut for further analysis. b) Analysis of the heart-cut in the analytical run. LE: 10 mmol/liter + β-alanine, pH 4.3 + 0.2 % w/v hydroxypropyl cellulose. TE: 10 mmol/liter acetic acid. Driving current 45 µA, chart speed 2.5 mm/s. Cl – chlorides, T – thiodiacetates. C – citrates. A – acetates. (c) Analysis of the heart-cut of the section between chlorides and phosphates in the analytical run. When TDA is to be determined, this section of zones togehter with the zone of phosphates is cut away and does not interfere. Conditions as in (b): LE: 10 mmol/liter HCl + β-alanine pH 4.3, 0.2 % w/v hydroxypropyl cellulose. TE: 10 mmol/liter acetic acid. Driving current 45 µA, chart speed 2.5 mm/s. Cl – chlorides, P – phosphates. A – acetates. In the upper part of (b) and (c) the derivative of the detector signal is recorded.

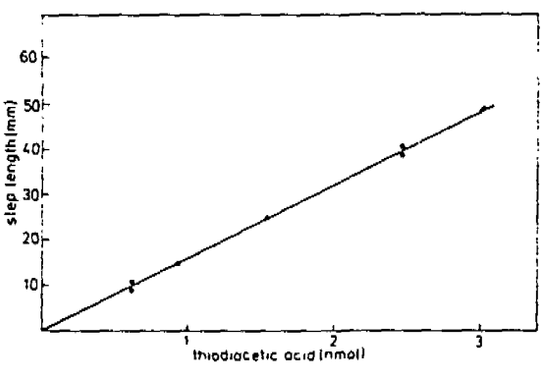

Figure 5. The calibration plot of the step-length in the record of the analytical run *vs.* the amount of TDA added to normal urine samples. The injected volume of urine with added TDA was 5 µl. Results were corrected to the amount of TDA present in urine before the standard was added. LE: 10 mmol/liter HCl + β-alanine + 0.2 % w/v hydroxypropyl cellulose, pH 3.4 and 4.3 in the pre-separation and analytical capillaries, respectively. TE: 10 mmol/liter acetic acid. Driving current: 200 µA and 45 µA, respectively. Chart speed for the analytical column 2.5 mm/s. Correlation factor 0.99992.

prolonged zone of chlorides, usually 5 µl of urine was analysed. Under the conditions described above, the step length of 1 mm in the record (with chart speed 2.5 mm/s) corresponded to 0.12 mm of the actual zone length in the analytical capillary and to the detection limit amounting to 0.061 nmoles of TDA. For the samples of 10 µl it represents the lowest detectable concentration of TDA being 6×10^{-6} mol/liter.

To determine the reproducibility of the method, the urine samples of 6 members of our laboratory staff and of 9 persons from the chemical workroom for PVC production were repeatedly analysed. The first group was considered not to be exposed to vinyl chloride and concentrations of TDA ranged within 0.025–0.067 mmol/liter. The values found for the second group varied between 0.075 and 0.15 mmol/liter. In both cases, the reproducibility of analyses was approximately the same and amounted to about 3 rel. %. The increase in the concentrations of TDA in urine of the persons exposed to vinyl chloride is evident.

4 Concluding remarks

TDA in urine of people both exposed and not exposed to VC can be reliably determined by analytical ITP with the column-coupling technique. The method proposed is rapid and samples of 10 µl of urine without any pretreatment can be directly analysed. The lowest detectable concentration of TDA was about 6×10^{-6} mol/liter. Reproducibility was 3 rel. %. The described method may also be the starting analytical procedure for the study of other anionic substances that appear in the urine of persons exposed to vinyl chloride.

Received March 16, 1984

R&S 005197

5 References

[1] Viola, P. L., Bigotti, A. and Caputo, A., *Cancer.Res.* 1971, *31*, 516–519.
[2] Müller, G., Norpoth, L. and Eckard, R., *Int. Arch. Occup. Environ. Health* 1976, *38*, 69–75.
[3] Müller, G., Norpoth, K., Kusters, E., Herweg, K. and Versin, E., *Int. Arch. Occup. Environ. Health* 1978, *41*, 199–205.
[4] Müller, G. and Norpoth, K., *Naturwissenschaften* 1975, *62*, 541.
[5] Sollenberg, J. and Baldesten, A., *J. Chromatogr.* 1977, *132*, 469–476.
[6] Vesterberg, O. and Sollenberg, J., *Excerpta Med. Int. Congr. Ser.* 1977, *No. 440*, pp. 186–189.
[7] Zschiesche, W., Schaller, K. H. ans Gossler, K., *Fresenius Z. Anal. Chem.* 1978, *290*, 115.
[8] Øvrebo, S., Jacobsen, D. and Sejersted, O. M., *3rd Internat. Symposium on Isotachophoresis*, June 1–4, 1982, Goslar, F.R.G., Symposium Abstracts, p. 69
[9] Sillén, L. G. and Martell, A. E., *Stability Constants of Metal Ion Complexes, Special Publication No. 17*, Chemical Society, London 1964.
[10] Harper, H. A., *Review of Physiological Chemistry*, (in Czech) Avicenum, Prague 1977, p. 453.
[11] Tschöpe, W. and Ritz, E., *J. Chromatogr.* 1980, *221*, 59–65.
[12] Everaerts, F. M., Verheggen, Th. P. E. M. and Mikkers, F. E. P., *J. Chromatogr.* 1979, *169*, 21–38.
[13] Deans, D. R., *J. Chromatogr.* 1981, *203*, 19–28.
[14] Boček, P., Deml, M., Kaplanová, B. and Janák, J., *J. Chromatogr.* 1978, *160*, 1–9.

Edwin H. McConkey
Catherine Anderson

Department of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, CO

Double-label autoradiography revisited: I. The ^{3}H and ^{35}S system

The method of double-label autoradiography described by McConkey (*Anal. Biochem.* 1979, *96*, 39–44) has been updated for the use of the commercial flour, EN^{3}HANCE, and the Kodak films XAR and SB. For gels 0.8 mm thick, the input ratio of ^{3}H dpm/^{35}S dpm should be 11.1 and the second-stage autoradiogram should be exposed 5.9 × as long as the fluorogram. For gels 0.4 mm thick, the input ratio of ^{3}H dpm/ ^{35}S dpm should be 5.3 and the second-stage autoradiogram should be exposed 3.1 × as long as the fluorogram.

Introduction

Comparison of two-dimensional gel patterns of complex protein mixtures, such as those obtained by O'Farrell's method [1], can be both difficult and ambiguous, due to unavoidable minor variations in the first and/or second stage, which cause even replicate samples to give patterns that are not perfectly superimposable. Several years ago, the senior author described a method of double-label autoradiography [2] which greatly facilitates the detection of one or a few differences in pairs of two-dimensional gel patterns, each of which may contain approximately 1000 co-migrating polypeptides. A sample containing ^{3}H-labeled proteins is mixed with a sample of ^{14}C - or ^{35}S -labeled proteins. Two-dimensional polyacrylamide gel electrophoresis is carried out and the gel is impregnated with a fluor to make detection of ^{3}H efficient. The dried gel is first autoradiographed on a film that records both ^{3}H and the higher energy isotope, via the production of light in the flour when beta particles are emitted. Then a second autoradiogram is made, using a film that is relatively insensitive to the flashes of light. Beta particles from ^{3}H are too weak to reach the film emulsion, but the stronger beta particles produced by ^{14}C and ^{35}S produce an image by direct interaction with silver halide crystals in the film emulsion. When the second stage autoradiogram is placed upon a photographic negative of the fluorogram, any spots that represent polypeptides labeled only with ^{3}H stand out as white spots, in contrast to the gray background of the negative and the detailed pattern of black spots representing ^{14}C or ^{35}S-labeled polypeptides.

Since the 1979 article was written, there have been several changes in the materials used for double-label autoradiography. First, the Kodak company has replaced XR film with XAR film and No Screen film with Direct Exposure film; second, SB film has been recognized as a desirable substitute for No Screen film in autoradiography without fluors (*e. g.*, [3]); and third, a commercially available fluor, EN^{3}HANCE (New England Nuclear) has largely replaced the use of PPO dissolved in dimethylsulfoxide for detection of ^{3}H in dried polyacrylamide gels. The purpose of this communication is to evaluate each of those new materials and to provide quantitative data that will enable experimenters to use them in double-label autoradiography with maximum efficiency.

2 Materials and methods

Calibration gels were made as described previously [2]. In brief, proteins labeled with ^{3}H, ^{14}C or ^{35}S were incorporated into 12 % acrylamide gels, which were then fixed, rinsed and impregnated with 20 % w/w diphenyloxazole (PPO) in dimethyl sulfoxide or with EN^{3}HANCE. Autoradiography was carried out at –70 °C or at room temperature (approximately 21 °C). Optical density of the film at 540 nm was measured in a Zeiss PMQII spectrophotometer, by holding a piece of film over the entrance slit to the sample chamber. All A_{540} values have been corrected for background, determined on a piece of the same film that was not in contact with a gel containing radioactivity. Values of A_{540} in excess of 1.2 were

Correspondence: Dr. Edwin H. McConkey, Molecular, Cellular and Developmental Biology, University of Colorado, Campus Box 347 Boulder, CO 80309, USA

Abbreviation: PPO: 2, 5, Diphenyloxazole

© Verlag Chemie GmbH, D-6940 Weinheim, 1984

0173-0835/84/0408-0230$2.50/0

R&S 005198