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Optimisation of a simultaneous separation of sulphonamides, dihydrofolate reductase inhibitors and β-lactam antibiotics by capillary electrophoresis

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Abstract

There are many high-performance liquid chromatographic assays for the determination of sulphonamides, dihydrofolate reductase inhibitors and β-lactam antibiotics individually in food products. Recently, some capillary electrophoresis (CE) assays for these drugs have appeared. Sulphonamides plus dihydrofolate reductase inhibitors are readily separated by capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) can separate β-lactams. However, if CE is to prove its value over HPLC techniques in practice then its intrinsic high resolution must be exploited fully. A combination of a sulphonamide–dihydrofolate reductase inhibitors separation by CZE with a MECC assay of β-lactams would be advantageous in the screening of food products for veterinary drug residues, saving both time and effort. The analysis of all three classes of drugs, in a single run, was tested using our established β-lactam MECC conditions, but this gave only a partial separation, with a very crowded region. A Box–Behnken factorial design was used to optimise this separation. It was carried out parallel with the "traditional trial and error procedure", based on our knowledge of the individual CE separations. Both methods arrived at a remarkably similar set of conditions. The final separation produced 25 peaks, within which, were 30 compounds, the separation taking 25 min.

Keywords: Factorial design; Optimization; Food analysis; Sulfonamides; Enzyme inhibitors; Lactams; Antibiotics

1. Introduction

Many veterinary drugs are used for the treatment of food-producing animals. Two commonly used classes of antibiotics are the sulphonamides and the β-lactams. Legislation [1] has set limits for the residues of these compounds in animal tissue, prompting the need for methods that can measure these residues. There are existing methods based on high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) to measure these compounds in animal tissue, although these are usually limited to a few compounds of one class per assay [2–5]. The high resolution of capillary electrophoresis (CE) offers the possibility of determining many compounds of different classes in a single rapid assay. This is advantageous, with there being a need to screen large numbers of samples. A procedure for measuring thirteen sulphonamides was described by Ackermans et al. [6] who showed that CE could be used for the analysis of pork meat samples.

Separation of standard antibiotics had previously been carried out using two different CE modes, the

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sulphonamides and dihydrofolate reductase inhibitors by capillary zone electrophoresis (CZE) [6–11] and the β-lactams by micellar electrokinetic capillary chromatography (MECC) [2,13–16]. Two methods were required due to the differing ionisation characteristics of the two classes of compounds. However, Dang et al. [17] separated a simple mixture of seven sulphonamides and trimethoprim using MECC conditions similar to the conditions already developed in our laboratory for the separation of β-lactams. This suggested to us the possibility of combining the sulphonamide and β-lactam assays. A single assay would make savings in time, effort and convenience. However, optimising the separation of such a complex mixture would itself be complex, but this could be simplified using chemometrics.

Advancements in statistical computing has made the application of chemometrics to the optimisation of chromatographic separations easier. Chromatographic separations offer many sets of conditions that give good resolution, but the goal is to locate the optimum maxima. This is not an easy task when there are many variables. It is often necessary to perform complex experiments, or to target the major factors; factorial designs are well suited to this application. [18,19]

Factorial designs have been frequently employed to optimise HPLC separations [18–22], but are only now being exploited for CE and, particularly, in MECC, which has many similarities with reversed-phase (RP)-HPLC [23].

A factorial design provides experimental data suitable for mathematical model fitting, thus making prediction possible. The experimental design can also be randomised to remove hidden effects that cannot be controlled. Factorial designs assume that all the variables are independent of each other. This is not always the case in CE and the data from a factorial design show whether the variables are independent of each other or whether interactions between variables occur. The extent to which interactions occur can be shown and this can be taken into account.

This paper shows the application of factorial design to the optimisation of a separation of a complex mixture of sulphonamides, dihydrofolate reductase inhibitors and β-lactam antibiotics, with a view to the determination of these compounds in animal tissue.

2. Experimental

2.1. Chemicals and reagents

The standard mixture initially consisted of 26 compounds (subsequently increased to 30) of three different classes of antibiotics.

2.1.1. Sulphonamides

The sulphonamides used were sulphanilic acid (SA), sulphanilamide (SAA), sulphacetamide (SAC), sulphaguanidine (SG,), sulphameter (SM), sulphamerazine (SMR), sulphamethazine (SMZ), sulphamethoxypyridazine (SMOP), sulphamethoxazole (SMOZ), sulphadiazine (SDI), sulphathiazole (ST), sulphapyridine (SP), sulphadimethoxime (SDIM), sulphisoxazole (SIOX) and sulphaquinoxaline (SQ). Succinyl sulphathiazole (SST) and phthalyl sulphathiazole (PST) were added later. All were obtained from Sigma–Aldrich (Poole, UK).

2.1.2. Dihydrofolate reductase inhibitors

Trimethoprim (TRI) and pyrimethamine (PY) were obtained from Sigma–Aldrich.

2.1.3. β-Lactams

The β-lactams used were moxacillin, ampicillin, piperacillin, penicillin G, oxacillin, penicillin V, cloxacillin, dicloxacillin and cephapirin. Nafcillin was added later. All were obtained from Sigma–Aldrich.

Levamisole (Sigma–Aldrich), although pharmacologically unrelated, was added later, because of its importance as a veterinary drug.

A standard mixture of the drug standards was prepared in deionised water, such that all of the drugs were at a concentration of 16 μg/ml.

Electrophoretic buffer solutions were prepared using sodium tetraborate (Merck, Lutterworth, UK), sodium dodecyl sulphate (SDS) and EDTA (Sigma–Aldrich). The pH was adjusted with 6 *M* HCl before addition of SDS to the buffer.

2.2. Instrumentation

All experiments were carried out using a Crystal 310 (Thermo Unicam, Cambridge, UK) controlled

with the Crystal CE control software, Version 1.3. The capillary was fused-silica (Polymicro Technologies, Phoenix, AZ, USA), 60 cm (47 cm to the detector)×50 μm I.D. The capillary temperature was 25°C. Hydrodynamic sample loading was for 3.6 s at 50 mbar (approx. 5 nl). Detection was performed using a Unicam 4225 tunable wavelength detector set at 205 nm. This was a compromise wavelength allowing detection of sulphonamides, dihydrofolate reductase inhibitors, β-lactams and levamisole.

Confirmation of the peak identities was carried out using a TSP 2000 (Thermo Separation Products, Stone, UK) with a scanning detector and spectral software capabilities.

2.3. Statistical methods

Statgraphic Version 6.0 program (Manugistics and Statistical Graphics, Rockland, MD, USA) was used to generate and manipulate the factorial design data.

3. Procedure

The previously reported MECC conditions of 25 m*M* phosphate–borate buffer containing 100 m*M* SDS, pH 8.5, and with a voltage of 12 kV had been used to separate sulphonamides and trimethoprim [17]. These conditions were similar to the β-lactam MECC separation conditions previously developed in our laboratory, i.e. 20 m*M* sodium tetraborate–75 m*M* SDS, pH 8.5, 24 kV, 210 nm. The viability of analysing all three classes of antibiotics in a single analysis using these conditions was tested. The voltage was reduced to 15 kV in order to improve the resolution and make the data easier to interpret for these investigative experiments. The chromatograms of these two groups of compounds are overlaid in Fig. 1. Fifteen peaks are visible, the dihydrofolate reductase inhibitors were not shown, since they eluted after 20 min. These initial studies showed the potential for analysing sulphonamides and β-lactams in a single run, but the optimisation procedure, due

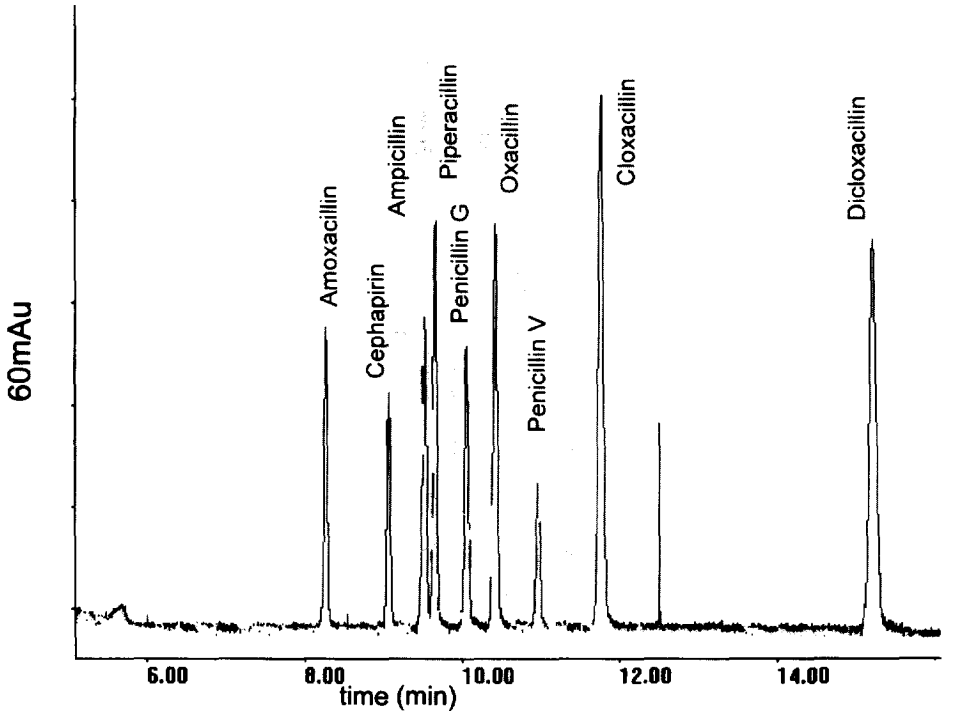

Fig. 1. Overlaid chromatograms of penicillins (black) and sulphonamides (grey) by MECC. Conditions: 20 m*M* sodium tetraborate–75 m*M* SDS, pH 8.5; 15 kV; 60(47) cm ×50 μm I.D. fused-silica capillary; 3.6 s 50 mbar hydrodynamic injection of 50 μg/ml standards; detection at 210 nm. For abbreviations see Section 2.

to the large number of variables in MECC, would be complex.

Berridge [18] proposed a three-level design for fitting response surfaces based on second order factorials. The designs are usually very efficient, requiring only a minimal number of runs.

The three factors chosen due to their importance in MECC were pH, voltage and SDS concentration [12]. Their significance was later confirmed from the data generated by the factorial design. The upper and lower limits were chosen to be 10.5 and 8.5, 30 and 10 kV, and 100 and 50 m*M*, for pH, voltage and SDS concentration, respectively; these were limited by the physical constraints of the instrument and buffer systems. The aim of the optimisation process was to maximise the number of peaks observed to 26.

The Box–Behnken model is depicted geometrically in Fig. 2. The fifteen points represent the fifteen experimental runs (Table 1).

The fifteen runs were performed and the number of peaks in each was counted (Table 1). These data were entered into the Statgraphic program, which produced a correlation of observed vs. predicted number of peaks (Fig. 3) to test the validity of the model. The R^2 value was 0.615. For a valid model, R^2 should be ≥ 0.6, indicating that predictions made using this mathematically fitted model were going to be accurate.

Following validation of the model, it was possible to produce response plots for the three different variable interactions. These surface response plots (Fig. 4a–c) show areas of interest, e.g. maxima in this experiment. Fig. 4a shows that low pH and high

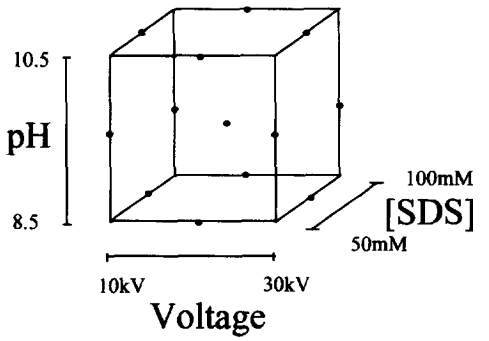

Fig. 2. A geometric representation of a Box–Behnken design for the three factors employed in this study.

Table 1
Experimental conditions and the number of peaks counted from the resulting chromatograms

Run	pH	[SDS] (m*M*)	Voltage (kV)	No. of peaks
1	9.5	75	20	17
2	9.5	100	30	16
3	9.5	50	30	13
4	10.5	75	10	9
5	10.5	100	20	18
6	10.5	50	20	23
7	8.5	100	20	20
8	9.5	75	20	17
9	8.5	75	30	21
10	9.5	100	10	22
11	8.5	50	20	17
12	9.5	50	10	20
13	8.5	75	10	21
14	10.5	75	30	15
15	9.5	75	20	17

[SDS] gave optimal conditions, Fig. 4b indicates high [SDS] was optimal as was high voltage and Fig. 4c shows that low pH and mid voltages were the optimum. The Statgraphics program gave these parameters different weightings, depending on the importance of the parameter interactions (Table 2), in which the greater magnitudes should have the greater physical effects. These data were then used by the Statgraphics program to predict the optimum conditions. These optimum conditions were pH 8.5, 100 m*M* SDS and 22 kV.

These predicted conditions were then employed

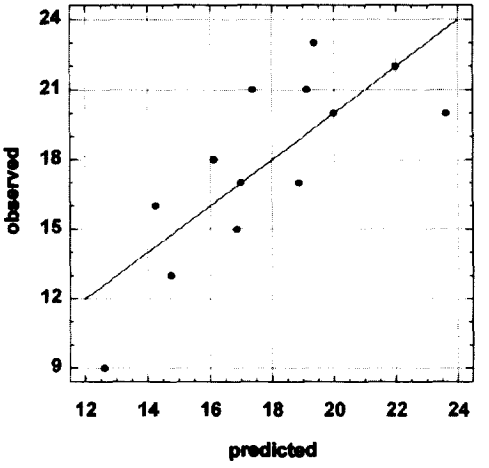

Fig. 3. Correlation plot of predicted vs. observed peak numbers.

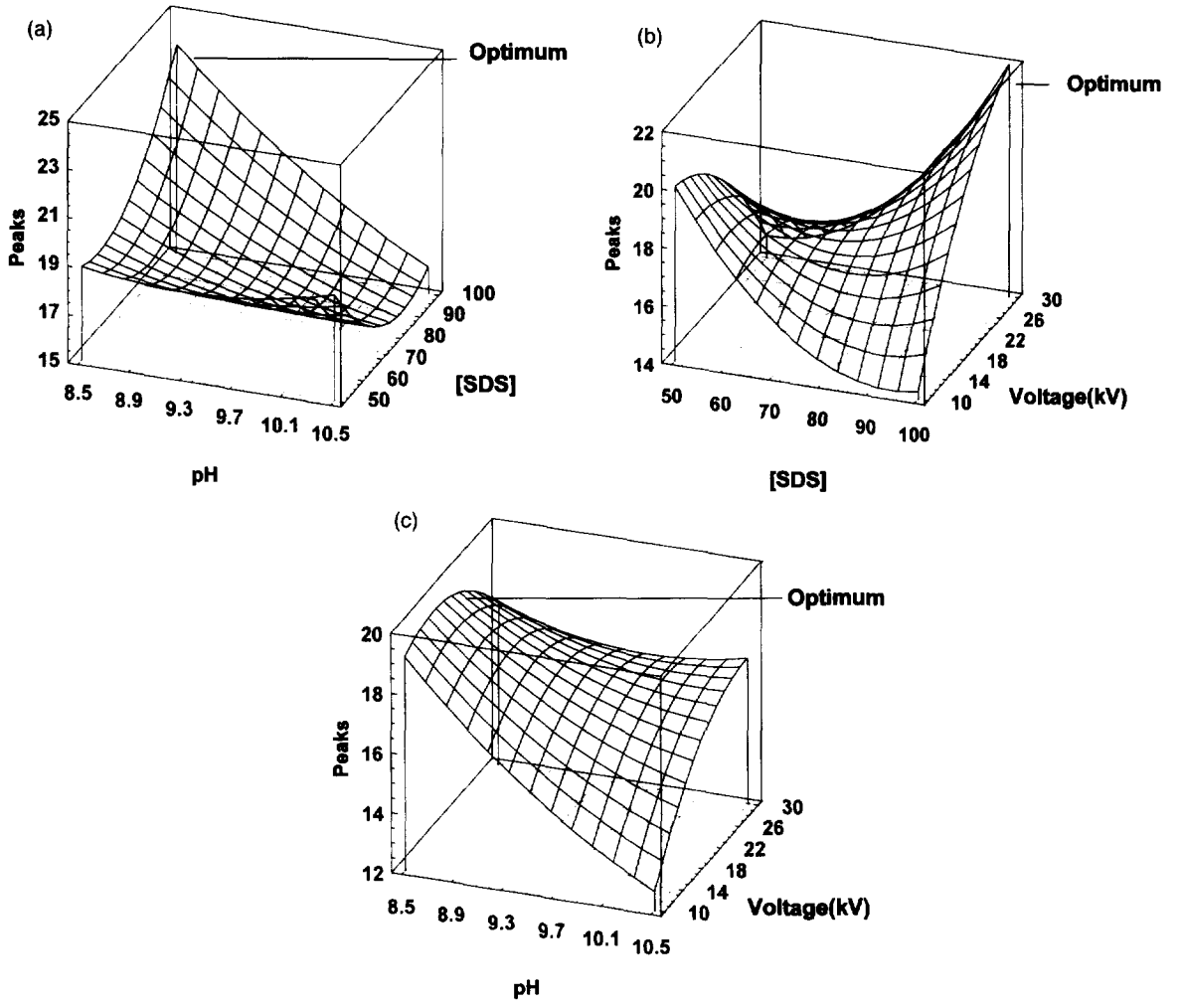

Fig. 4. (a) Response plot of pH vs. SDS concentration (m*M*). (b) Response plot of SDS concentration (m*M*) vs. voltage (kV). (c) Response plot of pH vs. voltage (kV).

Table 2
Parameter interactions

Parameter	Effect
pH	3.5
[SDS]	0.75
Voltage	1.25
pH and [SDS]	4
pH and voltage	3
[SDS] and voltage	6.5

for an actual separation and the resulting chromatogram is shown in Fig. 5. Twenty peaks can be seen, although many of them are grouped together at around 10 min and some are poorly resolved. However, the computer optimisation only used fifteen runs to evaluate the parameters and had to interpolate all the points therein, which is not infallible.

A parallel optimisation was carried out using traditional systematic optimisation techniques, using only the same three factors, i.e., pH, [SDS] and voltage. This was much more time-consuming and

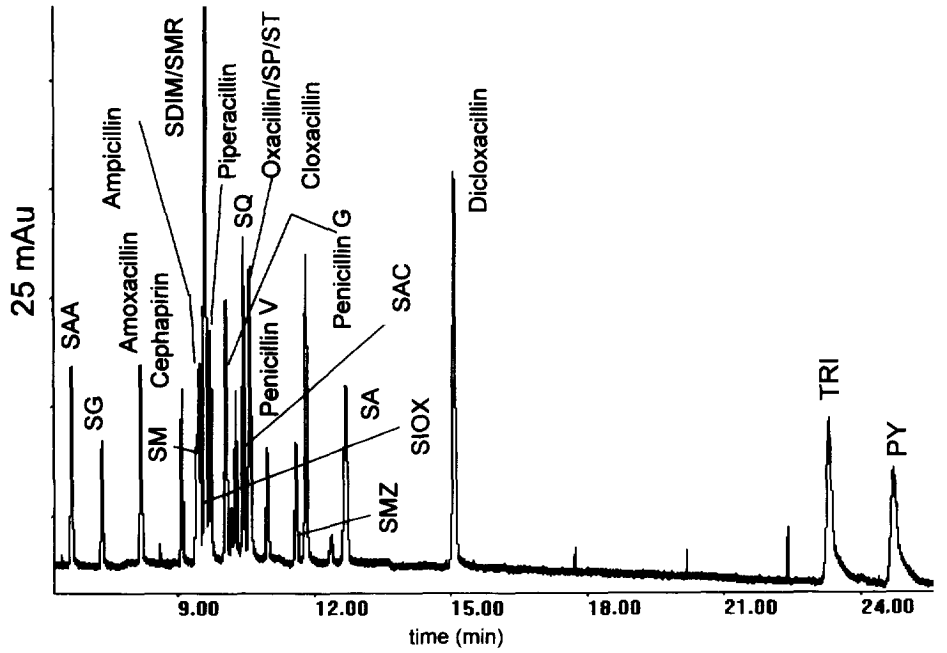

Fig. 5. Chromatogram of 26 sulphonamides, dihydrofolate reductase inhibitors and β-lactams using the computer-generated optimum conditions of 20 m*M* sodium tetraborate–100 m*M* SDS, pH 8.5; 22 kV; 60(47) cm×50 μm I.D. fused-silica capillary at 205 nm.

took many more runs (45 more than the computer optimisation) to achieve a sensible separation.

Slight tailing of some of the peaks was removed by adding 0.5 m*M* EDTA to the final running buffer (common practice in our laboratory; see Fig. 6, which also shows the additional compounds).

The final separation conditions were 20 m*M* sodium tetraborate–0.5 m*M* EDTA–100 m*M* SDS, pH 8.5, 15 kV; a 60 (47) cm×50 μm I.D. fused-silica capillary; 3.6 s 50 mbar hydrodynamic injection with detection at 205 nm.

4. Discussion

This investigation has shown us that it is possible to integrate a separation of sulphonamides and dihydrofolate reductase inhibitors and one of β-lactams in a single run by MECC. Figs. 5 and 6 show the chromatograms from the computer-generated conditions and from the systematic experimental optimisation. The conditions are remarkably similar, with only the voltage differing. The chromatograms are accordingly similar, with a slightly better separation at 22 kV. The penalty for this was a longer run time and loss in resolution of some peaks. The overlaid chromatograms indicate that seventeen peaks could be resolved; the computer-generated conditions producing eighteen peaks and the traditional optimisation method producing 21 peaks. The resolution of all of the compounds has not yet proved possible (30 compounds contained within 25 peaks), due to the co-elution of some sulphonamides and penicillins. The pH value of 8.5 used for the separation has not enabled the transference of the excellent sulphonamide separations [9].

The Box–Behnken factorial design optimisation has advantages over traditional methods; reduction in the number of experiments required, reduction in the time taken and generation of data that may be analysed statistically to provide valuable information on the interactions between experimental parameters, which may be useful for further optimisation (see Table 2).

This simultaneous separation could save a great deal of time, with one assay instead of two being

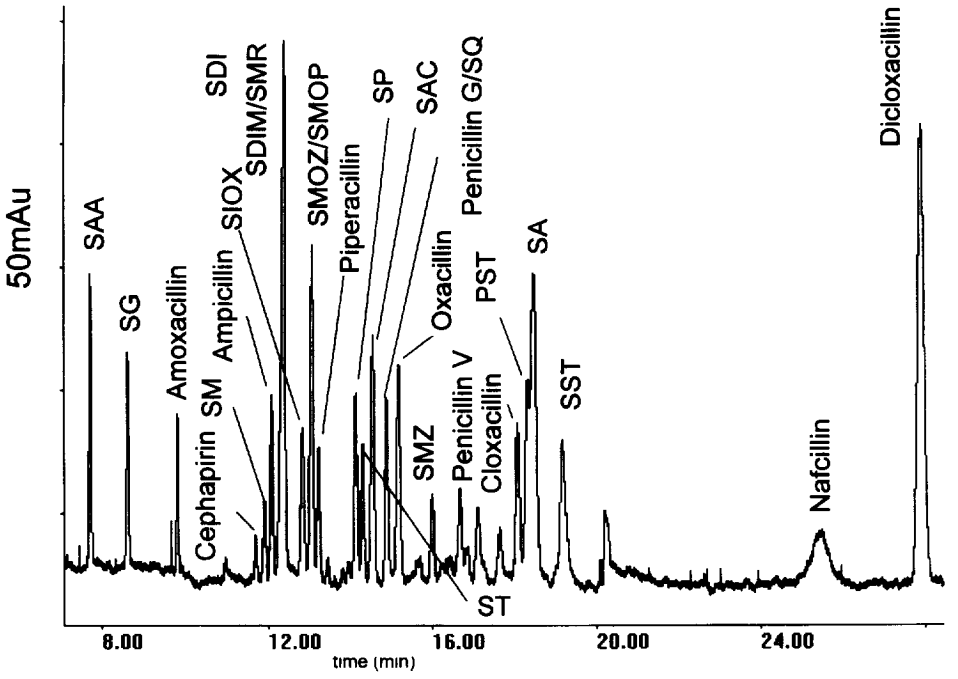

Fig. 6. Chromatogram of sulphonamides and β-lactams using the optimum conditions found by traditional methods, i.e., 20 m*M* sodium tetraborate–100 m*M* SDS–0.5 m*M* EDTA, pH 8.5; 15 kV; 60(47) cm×50 μm I.D. fused-silica capillary at 205 nm. (TRI, PY and levamisole elute after 24 min).

run. The time taken to develop this assay has been greatly reduced by the use of factorial design and, combined with some traditional optimisation, a very robust assay has been produced. This method will be of use for the simultaneous determination of sulphonamide, dihydrofolate reductase inhibitor and β-lactam residues in animal tissue.

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