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Optimisation of HPLC gradient separations using artificial neural networks (ANNs): Application to benzodiazepines in post-mortem samples

Rebecca Webb, Philip Doble*, Michael Dawson

Centre for Forensic Science, Department of Chemistry and Forensic Science, University of Technology Sydney, PO Box 123, Broadway, NSW, 2007, Australia

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ABSTRACT

Artificial neural networks (ANNs) were used in conjunction with an experimental design to optimise a gradient HPLC separation of nine benzodiazepines. Using the best performing ANN, the optimum conditions predicted were 25 mM formate buffer (pH 2.8), 10% MeOH, acetonitrile (ACN) gradient 0–15 min, 6.5–48.5%. The error associated with the prediction of retention times and peak widths under these conditions was less than 5% for six of the nine analytes. The optimised method, with limits of detection (LODs) in the range of 0.0057–0.023 μg/mL and recoveries between 58% and 92%, was successfully applied to authentic post-mortem samples. This method represents a more flexible and convenient means for optimising gradient elution separations using ANNs than has been previously reported.

1. Introduction

Optimising HPLC separations in order to obtain satisfactory resolution, sensitivity and analysis times can be a complicated and time-consuming process, given the large number of parameters that may be varied and the possibility of interactions between variables. Artificial neural networks (ANNs) are a multivariate approach that can be used for the optimisation of chromatographic separations. They are predictive data-processing programs modelled on the human brain, which have the ability to model and solve non-linear problems and discover the approximate rules that govern the optimal solution to these problems [1]. Unlike many other data-processing systems, which are programmed to arrive at the correct answer, ANNs are able to "learn" from a set of training examples that contain both the inputs and the desired outputs by plotting mapping functions. The relationships learnt using the mapping function from a particular data set can then be applied to new data, and predictions or generalisations can be made.

The basic processing unit of the ANN is the neuron, or node. In the most common type of ANN, the multilayer perceptron (MLP) feed-forward neural network, the network architecture is comprised of three layers of neurons; an input layer that receives the data, at least one hidden layer that processes the information received at the input, and an output layer, which is the observable response. Each neuron in the input layer is connected to each neuron in the hidden layer, and each one in the hidden layer is connected to each neuron in the output layer. The connections between the neurons are known as the weights. It is these weights that determine the behaviour of the ANN and how its behaviour changes over time during the learning process.

The error of a network is defined as the squared difference between the outputs and the target output values, summed over the number of output nodes and training patterns. The goal of training the network is to adjust the weights between layers in a direction so as to minimise the error. The most common type of training algorithm used in analytical applications is the backpropagation technique [2,3]. Using this technique, the training weights are initially given arbitrary values and an iterative approach is taken to find their optimal values. Each iteration is known as an epoch. Output nodes are informed of the target values and, based on the difference between the calculated output and the target output, the node determines the direction and the amount by which the weight has to move in order to minimise the error. The output nodes then propagate the amount of their errors to the hidden nodes, where it is used to determine in which direction and by how much the weights should change. After an appropriate number of iterations, the network will arrive at a minimum error, and training is stopped. At this point, the calculated outputs should be as close as possible to the experimental output values.

The biggest problem encountered when training ANNs is the tendency for over-learning or over-fitting, whereby the ANN does not describe the response adequately, despite the fact that the training data may fit well. As the network is trained, its mapping function becomes more complex, passing the point where generalisation is the best, on to over-fitting. The over-fitted ANN has a poor ability to generalise, or low predictive power. To prevent over-fitting, the data

* Corresponding author. Tel.: +61 2 95141792; fax: +61 2 95141460.
E-mail address: philip.doble@uts.edu.au (P. Doble).

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is split into a training set and a verification set. A good indication that over-fitting has occurred is when the verification error is significantly larger than the training error [2,3]. By watching the error on the verification set, training can be stopped before over-fitting occurs. Over-fitting can also occur when the training data contains noise. If too few data are used, the ANN will model the noise instead of the underlying features [4].

ANNs have been used to predict analyte retention times or resolutions in a number of isocratic HPLC separations [4–10], however their use in optimising gradient elution separations has been limited. Madden et al. [11] used an ANN to predict anion retention times in ion chromatography. The ANN inputs were three different gradient slopes, each having the same starting and finishing composition over various times. One potential disadvantage with this method is that slow gradients could result in long analysis times, which renders the optimisation process even more time-consuming. In addition, the optimum conditions were only selected from seven possible gradients. A similar method was employed by Buciński and Baczek [12] in which the optimum was selected from a set of only four gradient conditions. For the true optimum to be located, retention times should be predicted over all possible gradient conditions within the experimental space. In contrast, Shan et al. [13] used an ANN to predict analyte retention times under all possible pH and gradient conditions, and found the optimum conditions by calculating resolution function values at each point. However, the retention time of each analyte was modelled using a separate ANN which, for the separation of the seventeen amino acids in this study, required the construction of seventeen individual ANNs.

In this study, we report a flexible and convenient means for optimising the gradient elution separation of nine benzodiazepines using ANNs in conjunction with a simple experimental design to predict retention times and resolutions. The optimised method was validated and applied to the analysis of benzodiazepines in authentic post-mortem blood samples.

2. Materials and methods

2.1. Instrumentation

Experiments were performed on a Waters Alliance 2690 Separations Module, with Waters 996 Photodiode Array Detector (Waters, Sydney, Australia). All data manipulation was executed using Waters Millennium Software, Version 3.05. Separations were performed at 30 °C on a Zorbax SB-C18 column (50 mm × 4.6 mm, 1.8 μm) (Agilent Technologies, Sydney, New South Wales, Australia). The mobile phase was comprised of ammonium formate buffer (pH 2.80), acetonitrile (ACN) and MeOH at 1 mL/min. Duplicate 10 μL injections were performed and detection was at 280 nm. ANN modelling of each experimental space was performed using Trajan Neural Network Simulator Version 6.0 (Trajan Software Ltd, Durham, UK).

2.2. Reagents

All reagents were of analytical grade unless stated otherwise. Nitrazepam, oxazepam, alprazolam, flunitrazepam, temazepam and diazepam were obtained from Sigma–Aldrich (Sydney, New South Wales, Australia). 7-Aminoflunitrazepam, 7-aminonitrazepam and 7-aminoclonazepam were obtained from Novachem Pty Ltd (Melbourne, Victoria, Australia). Formic acid (HCOOH, APS Chemicals Ltd, Sydney, New South Wales, Australia) and ammonia solution (NH_4OH, APS Chemicals Ltd, Sydney, New South Wales, Australia) were used in the preparation of the mobile phase. Methanol (MeOH, Merck HPLC grade), ACN (Merck HPLC grade), ethyl acetate and diethyl ether were purchased from Crown Scientific (Sydney, New South Wales, Australia). MilliQ grade water

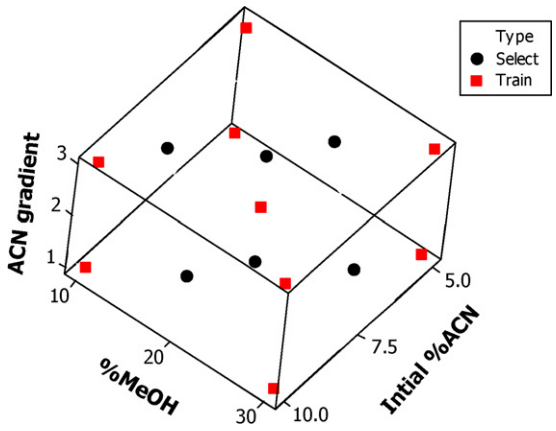

Fig. 1. Schematic of experimental space (train = training point; select = verification point).

(18.2 MΩ cm^{-1}) was used throughout the experiments. Buffers were prepared fresh each day and degassed by sonication prior to use. Whole sheep blood (Oxoid Australia Pty Ltd, Theberton, SA, Australia) was used for the preparation of spiked calibration standards. Prior to reconstitution, extracted samples were evaporated to dryness using a Heto VR Maxi vacuum concentrator (Medos, Sydney, New South Wales, Australia).

2.3. Calculations

The resolution of each peak pair (PP) was calculated as the difference between the retention times of each adjacent peak divided by half the sum of the peak widths. Separations were assessed according to the minimum resolution (R_{min}) between analytes in the separation. The relative error between experimental and predicted resolution and retention time values was calculated as the absolute value of the difference in the experimental and predicted values, as a percentage of the experimental value.

2.4. ANN modelling of the experimental space

Results of preliminary experiments with ammonium formate buffer indicated that buffer concentration and pH had little effect on the separation of the nine benzodiazepines, however selectivity varied depending on the choice of organic modifier. MeOH had some success at partially resolving all nine analytes, while ACN was shown to improve peak shape and overall analysis time. Therefore, an isocratic condition with respect to MeOH and a gradient condition with respect to ACN was implemented, giving an experimental design with the variables %MeOH and ACN gradient. %MeOH was defined as the isocratic percentage composition of methanol in the mobile phase. ACN gradient was defined as the percentage increase in acetonitrile composition divided by the duration of the linear gradient. In contrast to the other studies employing ANNs to model gradient separations, whereby the initial and final organic modifier concentrations were fixed, the initial composition of ACN (initial %ACN) was introduced as a third factor. This was done to give greater flexibility and to avoid the long analysis times required to generate slow gradients when initial and final compositions are known. Thus, the experimental space was as follows: 10–30% MeOH; 1–3%/min ACN gradient; 5–10% initial %ACN.

Fifteen experiments based on a central composite design were performed, of which nine were assigned as training points and six as verification (select) points, as illustrated in Fig. 1 and Table 1. Each experiment was performed in replicate with 10 μL injections of a mixed aqueous standard containing 10 μg/mL each of diazepam, nitrazepam, alprazolam, flunitrazepam, oxazepam, temazepam, 7-aminonitrazepam, 7-aminoflunitrazepam and 7-

Table 1
Values for each experiment in three factor design.

Experiment No.	ACN grad	%MeOH	Initial %ACN
1	1	20	7.5
2	2	10	7.5
3	3	20	7.5
4	2	30	7.5
5	2	20	10
6	2	20	5
7	2	20	7.5
8	3	30	10
9	3	10	10
10	1	30	10
11	1	10	10
12	3	30	5
13	3	10	5
14	1	30	5
15	1	10	5

aminoclonazepam. ANN architectures were constructed using an automated heuristic approach whereby the number of nodes in the hidden layer was varied. The most suitable ANNs were deemed to be the ones that had a sufficiently low training error with a corresponding verification error of the same magnitude, in order to minimise the likelihood of over-fitting. Data sheets containing a large number of possible points within the experimental space (10–30% MeOH at increments of 2%, 1–3%/min ACN gradient at increments of 0.1%/min, 5–10% initial ACN at increments of 0.5%) were created and the selected ANNs were applied to predict the output at each experimental point. In order to find the most appropriate ANN to model the chromatographic system, the effect of varying the output used as training data was investigated by comparing ANNs trained on resolution outputs to ANNs trained on retention time outputs. In addition, the effect of data quality and quantity was investigated by comparing ANNs trained on the data from each replicate to ANNs trained on the average data of both replicates.

2.5. Method validation

Calibration curves were obtained by analysing drug-free whole sheep blood, spiked with working standard solutions to obtain final concentrations of 0.432, 1.08, 1.44, 3.60 and 6.00 μg/mL for 7-aminonitrazepam, 7-aminoflunitrazepam and 7-aminoclonazepam, and 0.576, 1.44, 1.92, 4.80 and 8.00 μg/mL for oxazepam, nitrazepam, temazepam, flunitrazepam, diazepam and alprazolam. Calibration standards were analysed each day, and a standard curve constructed using linear regression. Accuracy and precision were calculated at high and low concentration for each drug, with five replicates at each concentration. Accuracy was expressed as the calculated concentration as a percentage of nominal concentration. Precision (%CV) was determined to be the percentage of the average divided by the peak area ratio of the three replicates. Recovery was calculated as the average peak area of each analyte in the spiked samples as a percentage of the average peak area of each analyte in aqueous standards. The limit of detection (LOD) was defined as a signal-to-noise ratio (*S*/*N*) of 3:1, and the limit of quantification (LOQ) was defined as a *S*/*N* of 10:1.

2.6. Sample preparation

The LLE method used for the extraction of benzodiazepines from whole post-mortem blood was based on a method reported previously [14]. Briefly, it involved the addition of 1 mL 2 M ammonia solution to 0.5 mL of whole blood, followed by 5 mL of diethyl ether/ethyl acetate (1:1, v/v). Each sample was vertically agitated for 2 min and centrifuged at 3000 rpm for 15 min. The organic layer was then transferred to a clean plastic tube, evaporated to dryness using a vacuum centrifuge and reconstituted in 100 μL MeOH prior to analysis.

The method was applied to post-mortem blood samples obtained from the Division of Analytical Laboratories (DAL) following coronial consent. All samples were preserved femoral blood taken as specimens at autopsy.

3. Results and discussion

3.1. ANN training

3.1.1. Resolutions as the output

Resolution between peaks is one of the more common outputs used when training an ANN to model chromatographic behaviour. For the first ANN, the values of the three factors and the corresponding replicate resolution data from the fifteen experiments were used as the inputs and outputs respectively, giving a total of eighteen training points and twelve verification points. After a thorough search through various ANN topologies, the ANN selected for the prediction of resolutions had a MLP architecture consisting of three input nodes, twelve hidden nodes and eight output nodes (resolutions for each of the eight peak pairs) (MLP 3:3-12-8:8). To determine the predictive ability of the selected ANN, the observed resolution values obtained from the fifteen experiments were plotted against the resolution values predicted by the ANN. Good correlation was obtained between the experimental and predicted resolution values ($r^2 = 0.9978$), as seen in Fig. 2, indicating good predictive ability of the ANN.

A second ANN was constructed using the average resolution values of each replicate. This halved the training and verification data, giving a total of nine training points and six verification points. Based on this method, the ANN that gave the best performance for the prediction of resolution values had a MLP architecture comprising of three input nodes, twelve hidden nodes and eight output nodes (MLP 3:3-12-8:8). As before, the observed average resolution values obtained for each analyte in the fifteen experiments were plotted against the predicted values generated by the ANN. Good overall correlation ($r^2 = 0.9968$) was found to exist between average experimentally obtained resolutions and these predicted resolutions.

Each ANN was applied to predict the peak pair resolution values throughout the experimental space. From this predicted data, three experimental points were selected and run to determine if the predicted data was consistent with this new experimental data. Both the ANN trained on replicate data and the ANN trained on average data demonstrated good ability to predict and learn the training data by virtue of their correlation coefficients, which were

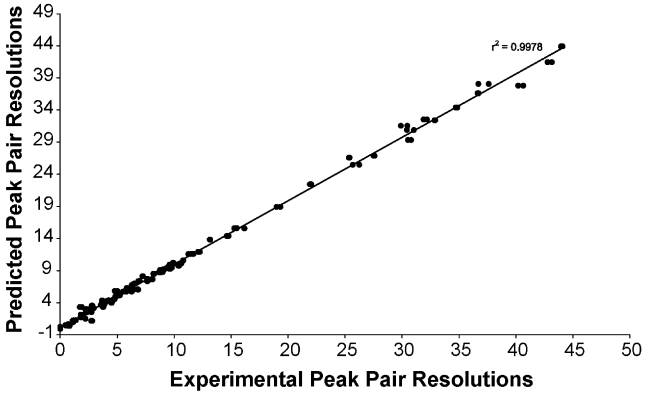

Fig. 2. Correlation between experimental and predicted resolutions using a MLP 3:3-12-8:8 ANN trained on replicate resolution data.

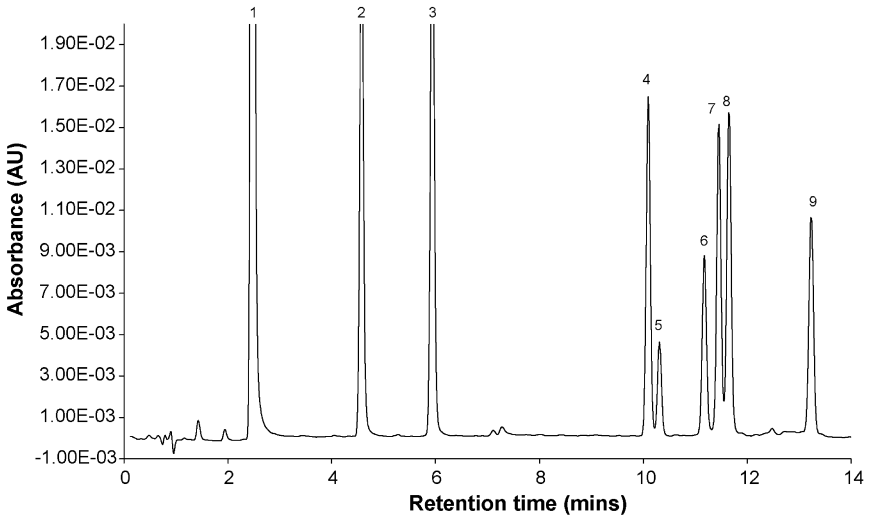

Fig. 3. Example chromatogram of the optimised separation of nine benzodiazepines. *Conditions*: Zorbax SB-C18 column (50 mm × 4.6 mm, 1.8 μm), temperature 30 °C, 10 μL injection, detection λ 280 nm, 25 mM formate buffer, pH 2.8, 10% MeOH, ACN gradient 0–15 min, 6.5–48.5%; *Elution order*: (1) 7-NH_2-NIT, (2) 7-NH_2-CLO, (3) 7-NH_2-FLU, (4) NIT, (5) OXA, (6) FLU, (7) ALP, (8) TEM, and (9) DIA.

greater than 0.99. However, the predictive ability of both networks diminished considerably when previously unseen data was presented. The ANN trained on replicate data had an average relative error of 13.8 ± 14.7% (range 0.752–51.9%), while the ANN generated using average resolution values had an average relative error of 13.2 ± 12.5% (range 0.453–46.8%) for the three experimental conditions tested.

3.1.2. Retention times as the output

Another common method employed when training ANNs to model chromatographic separations is to use retention time data as the output variable. For the first ANN, the training data comprised of the values of the three factors as the inputs, and the replicate retention time data for each analyte as the outputs, giving a total of thirty experimental points, of which twelve were assigned as verification points. The network that gave the best performance had a MLP architecture consisting of three inputs nodes, twenty hidden nodes and nine output nodes (retention times for each analyte) (MLP 3:3-20-9:9). Overall, the correlation between the experimental and predicted retention times was good (r^2 = 0.9981) suggesting good predictive ability of the ANN.

A second ANN was trained using the same experimental data, however the average retention times for each replicate were taken and assigned as outputs for the training and verification data, as opposed to considering each replicate individually. This gave a total of fifteen experimental data points, including six verification points. The best performing network comprised of three input nodes, twelve hidden nodes and nine output nodes arranged in a MLP architecture (MLP 3:3-12-9:9). As before, good correlation (r^2 = 0.9989) between experimental and predicted data was observed.

Both ANNs were applied to predict the retention times of each analyte throughout the experimental space. Three experimental points were then selected and run to determine if the predicted data was consistent with this new experimental data. The ANN trained on both replicates from each experimental point had an average relative error of 8.4 ± 9.7% (range 0.121–27.3%) when tested on the new data. The ANN trained on average retention data had a relative error of 9.3 ± 7.7% (range 0.787–25.6%).

Given the various outputs and types of training data that were considered, the best performing ANN was found to be the one trained on replicate retention time data. This network gave excellent overall correlation between experimental and predicted retention times for the training data set and, more importantly, it gave the lowest relative errors when presented with new data. This may have been due to the greater number of training cases that were available to this network as compared to the network that was trained on average data, and the fact that the retention times between each replicate showed very little variation i.e. the training data contained very little noise.

Unfortunately, knowledge of analyte retention times does not directly indicate the best separation, and resolutions between peak pairs must be determined. For this calculation, peak width data must be known. In this chromatographic system, the peak widths were found to vary considerably with the percentage of organic modifier in the mobile phase. An additional ANN was therefore constructed in order to predict the peak widths for each analyte throughout the experimental space. For this ANN, the values of each factor were again entered as inputs, while the average peak widths for each analyte comprised the nine outputs. Average data was used to eliminate the noise produced by slight variations in peak widths, giving a total of nine training points and six verification points. The best performing ANN for the prediction of peak widths had a MLP architecture consisting of three input nodes, twelve hidden nodes and nine output nodes (peak widths for nine analytes) (MLP 3:3-12-9:9). Correlation between experimental and predicted peak widths was satisfactory (r^2 = 0.9838). Three experimental points were selected and run to determine if the predicted data was consistent with the new experimental data. The average relative error was 7.7 ± 4.3% (range 0.282–19.1%).

3.2. Optimisation

The predicted retention times and peak widths generated by the two ANNs were used to calculate the resolution between each peak, and the optimum separation conditions were determined according to the minimum resolution (R_{min}). The data generated was sorted in order of descending R_{min} to identify the conditions for which R_{min} was a maximum. Ten different conditions gave rise to the maximum value of R_{min}, which was approximately 1.6, however each condition was associated with a different run time. From this data, and considering a maximum R_{min} and minimum run time, the optimum conditions were determined to be 10% MeOH, 6.5% initial ACN composition and a gradient of 2.8 (0–15 min, 6.5–48.5%). The ANN data predicted that these conditions would give resolution values greater than 1.5 and a run time of less than 13 min. To ascer-

Table 2
Predicted and experimental retention times and peak widths with corresponding errors for the optimum separation conditions.

Analyte	Predicted t_r (min)	Actual t_r (min)	Error (%)	Predicted width	Actual width	Error (%)
7-Aminonitrazepam	1.874	2.189	14.39	0.122	0.131	7.12
7-Aminoclonazepam	3.716	4.178	11.06	0.125	0.133	6.22
7-Aminoflunitrazepam	5.094	5.515	7.628	0.131	0.138	5.25
Nitrazepam	9.404	9.669	2.741	0.142	0.149	4.52
Oxazepam	9.618	9.908	2.927	0.128	0.130	1.89
Flunitrazepam	10.560	10.757	1.831	0.142	0.145	1.89
Alprazolam	10.890	11.044	1.394	0.140	0.143	2.31
Temazepam	11.130	11.247	1.040	0.155	0.157	1.41
Diazepam	12.850	12.845	0.039	0.153	0.155	1.49

tain that these conditions were indeed the optimum, they were verified experimentally, and an example of the separation attained is illustrated in Fig. 3. The predicted and experimental retention times and peak widths, with associated relative errors under these conditions are illustrated in Table 2. The errors concerning the prediction of retention times at the optimum ranged from 0.039% to 14.39%, with an average of 4.78%. The largest error was associated with the prediction of the retention time of 7-aminonitrazepam. Even though the absolute difference between the experimental and predicted retention times for this analyte was only 0.315 min, this equated to a large percentage error since 7-aminonitrazepam had the least retention on the column. The errors relating to the prediction of peak widths at the optimum ranged from 1.41% to 7.12%, with an average of 3.57%. Again, the largest error was associated with the prediction of 7-aminonitrazepam. According to Zakaria et al. [15], predictive errors using ANNs, or indeed any other model, should be less than 5% for optimisation purposes. The results for six of the nine analytes are in agreement with this. While higher than desired, the errors associated with the prediction of the 7-amino metabolites had no bearing on the final outcome since these analytes were well-resolved under all conditions, including the optimum.

Table 3 illustrates the predicted and experimental resolution values, which were calculated using the predicted and experimental retention times and peak widths. Unfortunately, a substantial error was associated with the prediction of the resolution between alprazolam and temazepam (PP7), which was the most difficult peak pair to separate. So while baseline resolution was predicted (R_s = 1.63), this was not observed when the conditions were run, and a resolution value of only R_s = 1.35 was obtained experimentally. Error in the predicted resolution values could be due to the accumulation of small errors in predicted retention and peak width data, which must be added together when retention times and widths are inputted into the resolution equation.

Table 3
Predicted and experimental resolution values for the optimum separation conditions.

	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8
Predicted	14.9	10.8	31.6	1.59	6.98	2.34	1.63	11.2
Experimental	15.1	9.87	29.0	1.71	6.17	1.99	1.35	10.2

PP1 = 7-Aminonitrazepam/7-Aminoclonazepam; PP2 = 7-aminoclonazepam/7-aminoflunitrazepam; PP3 = 7-aminoflunitrazepam/nitrazepam; PP4 = nitrazepam/oxazepam; PP5 = oxazepam/flunitrazepam; PP6 = flunitrazepam/alprazolam; PP7 = alprazolam/temazepam; PP8 = temazepam/diazepam.

Table 4
Recovery of benzodiazepines from whole sheep blood.

Analyte	Concentration (μg/mL)	Recovery (%, mean ± S.D.)
7-Aminonitrazepam	6.0	58 ± 5
7-Aminoclonazeapm	6.0	64 ± 1
7-Aminoflunitrazepam	6.0	61 ± 4
Nitrazepam	8.0	77 ± 8
Oxazepam	8.0	86 ± 11
Flunitrazepam	8.0	92 ± 20
Alprazolam	8.0	75 ± 6
Temazepam	8.0	75 ± 6
Diazepam	8.0	75 ± 13

The optimisation of gradient elution separations using ANNs has been previously reported, however the method presented here offers a number of advantages. Firstly, in contrast to the methods presented by Madden et al. [11] and Shan et al. [13], the initial and final compositions of the organic modifier do not need to be constrained. Instead, the initial composition of organic modifier and the slope of the gradient can be used as input variables for an ANN, and the final composition can be calculated from the gradient slope after substituting in an appropriate time. Not only does this allow for greater flexibility because it does not rely on fixed starting and finishing compositions, but it also effectively opens up the experimental space and increases the possibility of finding the optimum separation conditions. In addition, having flexible starting and final concentrations avoids the use of long analysis times that are required to implement slow gradients when concentrations are fixed. Another advantage of this method is that separate ANNs do not need to be constructed for each analyte, unlike the method presented by Shan et al. [13].

3.3. Method validation

Calibration curves for spiked whole blood were linear in the range 0.432–6.00 μg/mL for 7-aminonitrazepam, 7-aminoflunitrazepam and 7-aminoclonazepam, and 0.576–8.00 μg/mL for oxazepam, nitrazepam, temazepam, flunitrazepam, diazepam and alprazolam. Correlation coefficients (r^2) ranging from 0.9973 to 0.9993 were established in these ranges. The LODs were between 0.0057 μg/mL (nitrazepam) and 0.023 μg/mL (flunitrazepam). Intra-assay precision and accuracy were determined by analysis of five replicate samples at high and low concentration within the same validation batch. Inter-assay precision and accuracy were assessed by analysing five replicate samples at high and low concentrations. Intra-assay precision was between 0.18% and 3.0%, while inter-assay precision was between 0.15% and 13%. Accuracies were greater than 97%, while recoveries were between 58% and 92% as shown in Table 4.

Table 5
Blood concentrations of benzodiazepines in post-mortem blood samples.

Case no.	Diazepam	Temazepam	Oxazepam
1	1.2	4.0	BLOQ
2	1.4	0.12	N.D.
3	N.D.	N.D.	4.6
4	N.D.	N.D.	1.8
5	0.3	N.D.	N.D.

Abbreviations: BLOQ, below limit of quantification; N.D., not detected.

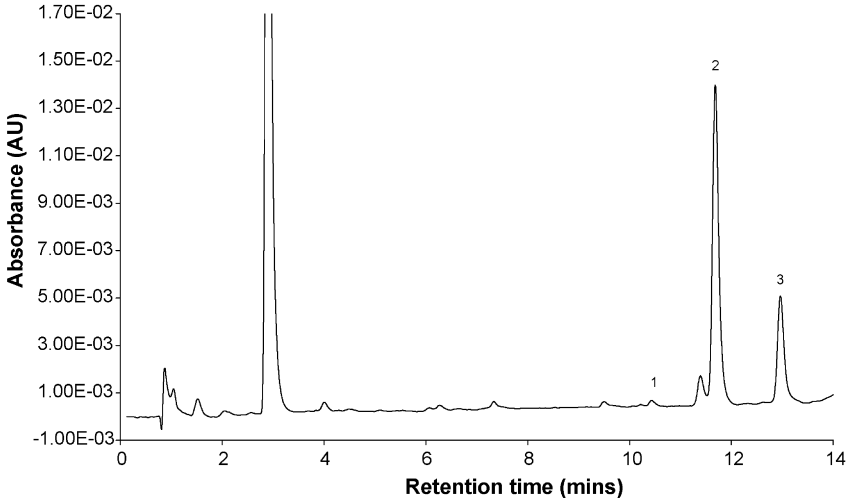

Fig. 4. Example chromatogram for post-mortem blood sample. *Conditions*: Zorbax SB-C18 column (50 mm × 4.6 mm, 1.8 μm), temperature 30 °C, 10 μL injection, detection λ 280 nm, 25 mM formate buffer, pH 2.8, 10% MeOH, ACN gradient 0–15 min, 6.5–48.5%; *Elution order*: (1) OXA, (2) TEM, and (3) DIA.

3.4. Method application

Five post-mortem blood samples were analysed and the benzodiazepine concentrations found in each sample are reported in Table 5. Three of the samples were found to contain diazepam in the range 0.3–1.4 μg/mL; two samples contained temazepam at concentrations of 0.12 and 4.0 μg/mL; and two samples had quantifiable levels of oxazepam at 1.8 and 4.6 μg/mL. Oxazepam was detected at sub-therapeutic concentrations in an additional case, however the concentration was below the limit of quantification (BLOQ). An example chromatogram of this case can be seen in Fig. 4. The sensitivity of the method was such that it enabled the quantification of benzodiazepines found at fatal, toxic and even therapeutic concentrations in post-mortem samples. In one case, the concentration of oxazepam was BLOQ, however the method was still sensitive enough to enable its detection.

4. Conclusions

A combination of a three factor experimental design and ANNs was applied to the optimisation of a gradient elution HPLC separation of nine benzodiazepines. Following investigation of a number of ANNs, the best-performing one was found to be trained on replicate retention time data, and the optimum conditions predicted were 25 mM formate buffer (pH 2.8), 10% MeOH, and ACN gradient 0–15 min, 6.5–48.5%. The error associated with the prediction of retention times and peak widths under these conditions was less than 5% for six of the nine analytes studied. This method represents a more flexible and convenient means for optimising gradient separations than has been previously reported. The optimised method was validated for blood and successfully applied to authentic post-mortem samples. The limits of detection of the method ranged from 0.0057 to 0.023 μg/mL, and recoveries were in the order of 58–92%.

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