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Development of a liquid chromatography–tandem mass spectrometry method for an euthanasic veterinarian drug: Tanax®

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ABSTRACT

A development of a rapid and sensitive LC–MS/MS method for the simultaneous detection of active ingredients of the euthanasic veterinarian drug Tanax® mixture is described.

The method proposed, with a retention time of few minutes (6 min) was developed for an equine serum sample with solid-phase extraction (S.P.E). This S.P.E. procedure has been revealed useful for the determination of very low concentrations of Tanax® analytes (0.05–1 ng/ml).

The method was validated in terms of specificity/selectivity, sensitivity, recovery and precision.

1. Introduction

Among the commercially available non-inhalant veterinarian formulations for euthanasia, Tanax® is one of the best known [1–4]. For its narcotic and curariform-like activity is commonly used in veterinary medicine [5,6]. As far as it is known on the basis of literature, it is composed by three substances in a DMF–water solution (*N*,*N*-dimethyl-formamide and water 6:4 v/v). Tanax® (T-61) is a mixture consisting of these three compounds: *N*-2-ethyl-2-(3-methoxyphenyl)-butyl-4-hydroxy-butanamide or embutramide (200 mg ml^{-1}), 4,4′-methylene bis-*N*,*N*,*N*-trimethylcyclohexanaminium di-iodide or mebenzonium iodide (50 mg ml^{-1}) and *p*-butylaminobenzoyl-dimethylamino-ethanol chloride or tetracaine hydrochloride (5 mg ml^{-1}) (Fig. 1) [7].

The strong narcotic effect and deepness anaesthesia by paralysing brain centres that control breathing in central nervous system is due to the general anaesthetic embutramide [8].

Curariform-like action is exerted by mebenzonium iodide, this paralyses the skeletal muscles and quite immediately induces a respiratory collapse cause of a paralysis of intercostals muscles and of the diaphragm [9].

In view of its local anaesthetic activity for reducing painful tissue reactions at the injection site, tetracaine hydrochloride is used in T-61 mixture [10].

Frequently Tanax® has been involved in deaths or suicide attempts of human beings [11–13] as well as in cases of malicious death in apparently healthy animals such as horses.

Medical diagnosis can be assisted by a quick analytical determination of Tanax® components in human or animal biological tissues or fluids. For reaching this purpose different analytical methods for the determination of embutramide have been applied: gas chromatography (GC) coupled with mass spectrometry (MS) [11,14,15] and high-performance liquid chromatography coupled with UV [16,17]. On the contrary, just few methods are described in literature for mebenzonium iodide determination [18,19].

This is probably caused by the difficulty to identify it in biological materials due to its low solubility in most of the common solvents [18]. Furthermore, because of its quick degradation by esterase enzyme, tetracaine hydrochloride has been difficult to detect jet.

From the literature it is clear that the most important problem derived from the use of this euthanasian veterinarian drug is connected with the identification into biological tissues and fluids of the three components and solvent of Tanax®. Till now, analytical methods for the simultaneous determination of the three components are not developed jet. Furthermore, no evidence of LC–MS/MS methods has been presented.

In the same time even forensic science is focusing the attention on the simultaneous identification of Tanax® mixture to confirm the hypothesis of intoxication in humans or suicide attempts.

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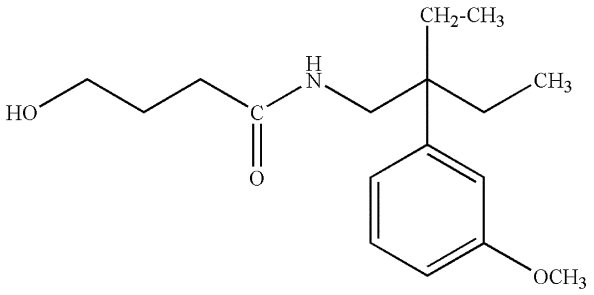
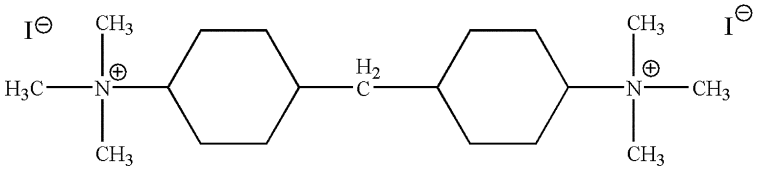


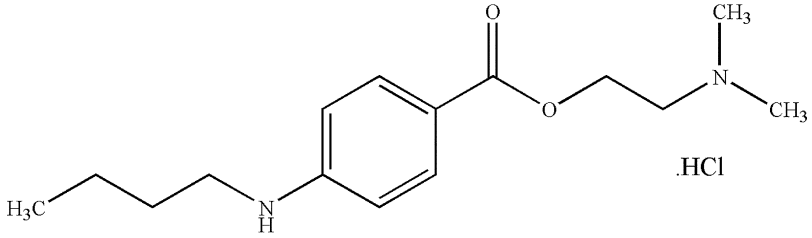
N-ethyl-2-(3-methoxyphenyl)-butyl-4-hydroxybutanamide

Embutramide



4,4'-methylene bis-N-N,N-trimethylcyclohexanaminium diodide

Mebezonium Iodide



p-butylaminobenzoyl-dimethylamino-ethanol chloride

Tetracaine hydrochloride

Fig. 1. The three compounds of Tanax®.

The aim of this study was to develop a LC–MS/MS method for the detection of active ingredients of Tanax® mixture in biological equine serum.

The method proposed, with a retention time of few minutes, was developed for a matrix (serum) after solid-phase extraction (S.P.E). This S.P.E. procedure has been revealed useful for the determination of low concentrations (0.05–1 ng/ml) of Tanax® analytes.

A real case of a suspected Tanax® induced death horse was reported with the method studied above.

Quality parameters of the method proposed were determined and discussed. The method described was validated in terms of specificity/selectivity, sensitivity, recovery and precision.

The main advantages, rapidity, selectivity and sensitivity of the studied LC–MS/MS method could in a near future revile it as a valid instrument for a rapid determination of Tanax® utilization.

2. Experimental

2.1. Reagents

Embutramide and mebenzonium iodide were directly obtained by the veterinarian speciality Tanax® purchased from Intervet International GmbH. Tetracaine, lidocaine and ipratropium bromide were purchased from Sigma–Aldrich. While *N,N*-dimethylformamide was purchased from Fluka.

Methanol, trifluoroacetic acid 99.5%, hexane, 2-Propanol and water of analytical grade were purchased from J.T. Baker, ammonium acetate from Merck.

2.2. Preparation of stock solutions

Individual stock solutions of tetracaine, lidocaine and ipratropium bromide (using standard powders) were prepared at a concentration of 1 mg/ml, by dissolving 1 mg of each compound in 1 ml of methanol.

The working solutions at a concentration of 1 μg/ml were prepared using an intermediate stock of 10 μg/ml (obtained by diluting 100 μl of the individual stock solution in 10 ml of methanol) from which 1 ml was diluted in to 10 ml of methanol.

For embutramide and mebenzonium, because of the absence of standard powders, separate working solutions were prepared diluting in methanol a proper quantity of veterinarian speciality Tanax® in which 200 mg/ml of embutramide and 50 mg/ml of mebezonium are present.

To obtain a final concentration of 1 μg/ml of embutramide and mebezonium, 50 and 200 μl of veterinarian speciality were

diluted in 10 ml of methanol (intermediate concentration of 1 mg/ml), respectively. Successively, 100 μl of each intermediate stock solution containing 1 mg/ml were diluted in 10 ml of methanol obtaining a main stock solution of 10 μg/ml. Finally, the concentration of 1 μg/ml was obtained by dilution of 1 ml of the each solution in 10 ml of methanol.

2.3. Preparation of samples

Appropriate volumes (100 μl) of working solutions were added to 2 ml of serum to give an appropriate concentration of 50 ng/ml for each compound. At the same concentration, the lidocaine and ipratropium bromide, used as internal standards were added.

These samples obtained were then treated by S.P.E. extraction and subsequently LC–MS/MS analysis were performed.

2.4. Instrumentation

An LTQ linear ion-trap mass spectrometry equipped with an ESI source (Thermo-Fisher, San Josè, CA, USA) connected to a Surveyor Autosampler-MS Pump (Thermo-Fisher) was used.

Chromatographic separation was achieved on a Ciano-phase Luna™ column (150 mm × 2 mm, 5 μm; Phenomenex, San Josè, CA, USA) equipped with a 2 mm i.d. × 4 mm ciano guard column.

Compound identities were confirmed in the positive electrospray ionization (ESI) MS^n mode, with full-scan product acquisition in the *m*/*z* 50–300 range.

In order to obtain data, the Excalibur® software (version 1.4, Thermo-Fisher) was used.

2.5. Method

2.5.1. Sample treatment

For the protein precipitation, a 2 ml serum sample was placed in a 10 ml glass tube and added with 2 ml of methanol. The tube was vortexed and than centrifuged at 2500 rpm for 10 min. The supernatant was transferred to a different extraction tube, where HCl (0.2 M) was added for reaching pH 6 and subsequent filtered with a 0.45 μm nylon membrane (30 mm, Chromacol Ltd., Welwyn Garden City, United Kingdom). Then, 100 μl of the two internal standards, lidocaine and ipratropium bromide at the concentration of 1 μg/ml, were added.

2.5.2. Solid-phase extraction

The samples were extracted with STRATA-X-CW (500 mg, Phenomenex, San Josè, CA, USA) cartridges and these were conditioned with methanol (5 ml) followed by 5 ml of 0.02 M acetate buffer, pH 6. The sample was then passed slowly through the column. The cartridge was rinsed with 2 ml of 0.02 M acetate buffer (pH 6), followed by 3 ml hexane allowed to dry for 5 min under vacuum.

Analytes were eluted with a mixture (5.8 ml) of methylene chloride/isopropanol/formic acid 70:20:10, respectively. The eluate was evaporated to dryness under gentle nitrogen stream and 100 μl of mobile phase were added in order to resuspend the residue. Five microliters of the resulting solution was injected into the LC system.

2.5.3. LC and ESI–MS/MS conditions

The LC separation of Tanax® active ingredients was achieved using an isocratic elution. The mobile phase used in LC–MS/MS was composed of 60% trifluoroacetic acid 0.1% (solvent A) and 40% methanol (solvent B). The flow rate of the mobile phase was maintained at 0.25 ml/min. The injection volume was 5 μl.

Under these conditions, the LC run time for each injection was approximately 6 min. LC–MS/MS chromatogram from a spiked equine urine sample with Tanax® ingredients is shown in Fig. 2. This acquisition method led to the identification and quantification of the three different components.

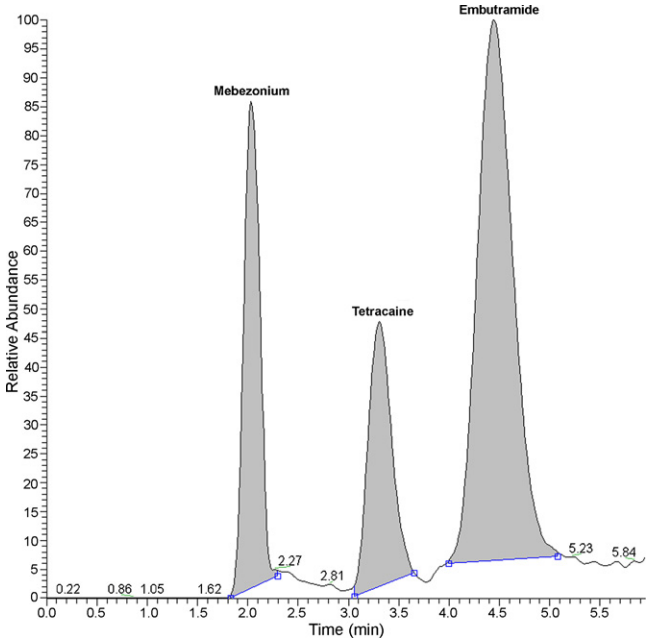


Fig. 2. Ion current profiles obtained from LC–MS/MS analysis of an equine urine spiked with Tanax® active ingredients.

The choice of mobile phase was justified by the mebezonium peak resolution, in fact the trifluoroacetic acid withdraw the secondary interactions with the column.

The ESI–MS/MS conditions were first optimized by direct injection (flow injection analysis) of each compound individually at concentration of 10 μg/ml at a flow rate of 20 μl/min. Cause of high selectivity of ESI MS/MS a direct injection of diluted Tanax® veterinarian speciality could be possible, this permitted the characterization of mebezonium and embutramide.

All the analysis use the positive electrospray ionization (ESI^+) MS^n mode, with full-scan product acquisition in the *m*/*z* 50–300 range. A capillary voltage of 21 V, a capillary temperature of 270 °C and a spray voltage of 8 kV were employed; the nitrogen sheath and auxiliary gas flow rates were set at 20 and 10 arbitrary LTQ units, respectively.

Data acquisition for MS^n was performed in 4 segments: the 1st (0–2.15 min.) with 2 scan events for mebenzonium iodide, the 2nd (2.15–2.90 min) with 2 scan events for ipratropium bromide and lidocaine, the 3rd (2.90–4.0 min) for tetracaine and the 4th (4.0–6 min) for embutramide. The collision energy was 24% for mebenzonium iodide and ipratropium, 23% for lidocaine and tetracaine and 22.5% for embutramide.

Major ions in LC–MS/MS full-scan product ion spectra for the active ingredients of Tanax® and for I.S. used are shown in Table 1. In Fig. 3, the extracted ion chromatograms and relative mass spec-

Table 1
Major ions in LC–MS/MS full-scan product ion spectra for the active ingredients of Tanax® and for I.S. used

Compound	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)
Mebezonium iodide	148	60, 236
Lidocaine	235	86
Ipratropium bromide	332	124, 166, 290
Tetracaine hydrochloride	265	176, 220
Embutramide	294	121, 135, 149, 191, 208, 276

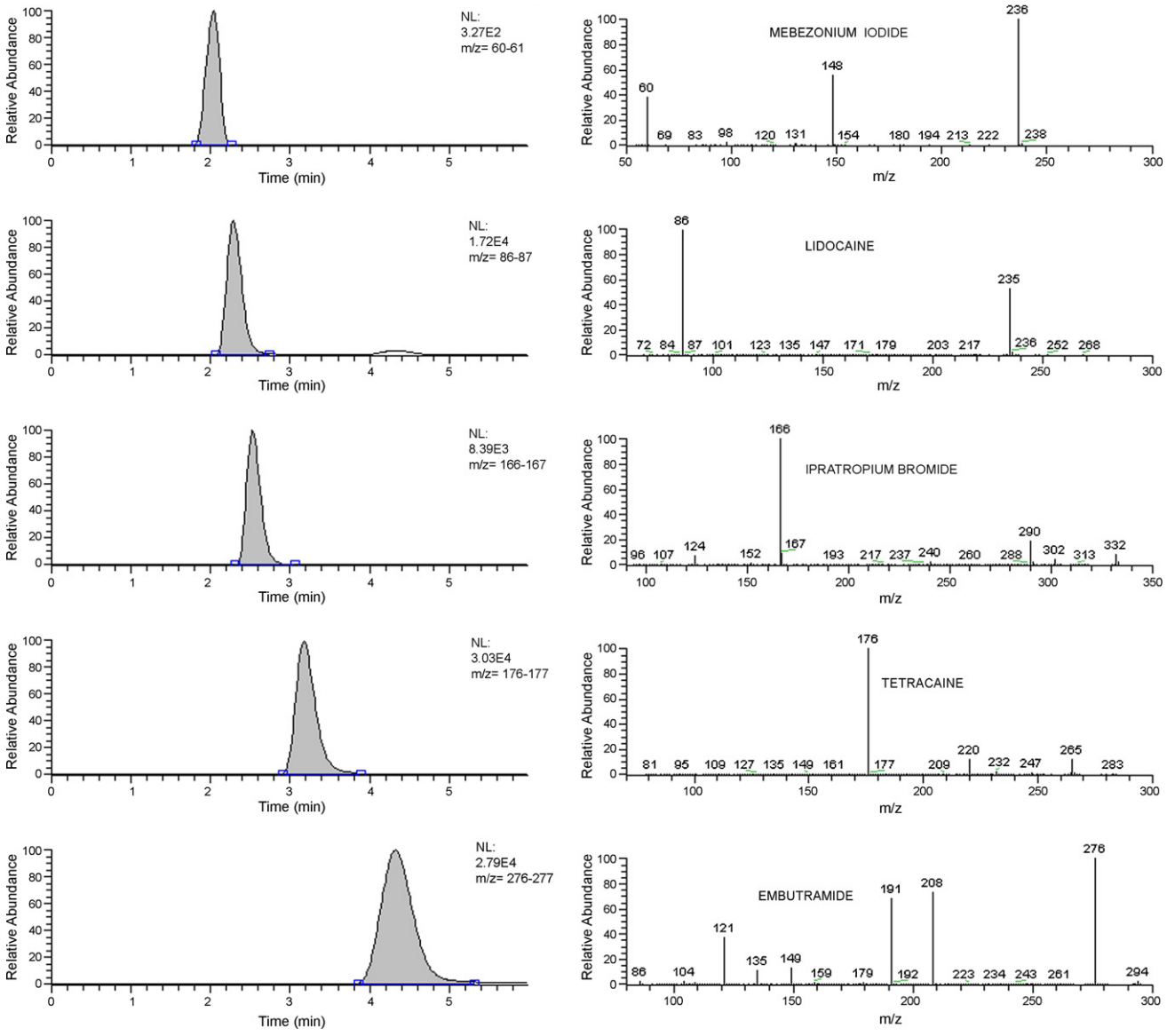


Fig. 3. Extracted ion chromatograms of *m*/*z* 60 (MEBENZONIUM IODIDE), *m*/*z* 86 (LIDOCAINE), *m*/*z* 166 (IPRATROPIUM BROMIDE), *m*/*z* 176 (TETRACAINE), *m*/*z* 276 (EMBUTRAMIDE) and their relative full-scan LC–MS/MS spectra.

tra obtained from LC–MS/MS analysis of a mixture composed by internal standards (lidocaine and ipratropium) and Tanax® active ingredients are reported.

3. Results and discussion

3.1. Validation parameters

3.1.1. Specificity/selectivity

Selectivity or specificity is the ability of a method to determine accurately and specifically the analyte of interest in presence of other components in a sample matrix under the studied conditions.

To test the specificity, five control blank serum samples were analysed using the proposed method. The resulting chromatograms were compared with those obtained from equine serum samples. No significant interferences or endogenous peaks at the retention time of the analytes or internal standards were found, as it can be shown in Figs. 4 and 5.

3.1.2. Sensitivity

The limit of quantification (LOQ) of each compound was considered as the concentration giving a signal to noise ratio of 10. The limit of detection (LOD) was defined as 0.3 × LOQ.

For the Tanax® componets LODs and LOQs are represented in Table 2.

3.1.3. Extraction recovery

Extraction recovery is a measure of the efficiency of the extraction of the analyte from the sample matrix. It is expressed as the ratio of the response obtained when the analyte is submitted to the extraction procedure to that measured when it is determined without the extraction step. The extraction recovery was studied by analysis of five replicates of a positive control samples, blank serum samples spiked with analytes at the concentration of 5, 50 and 500 ng/ml for each analyte. The response obtained with these samples was compared to that obtained with samples where the analytes were added after extraction of the blank matrix, representing 100% extraction recovery.

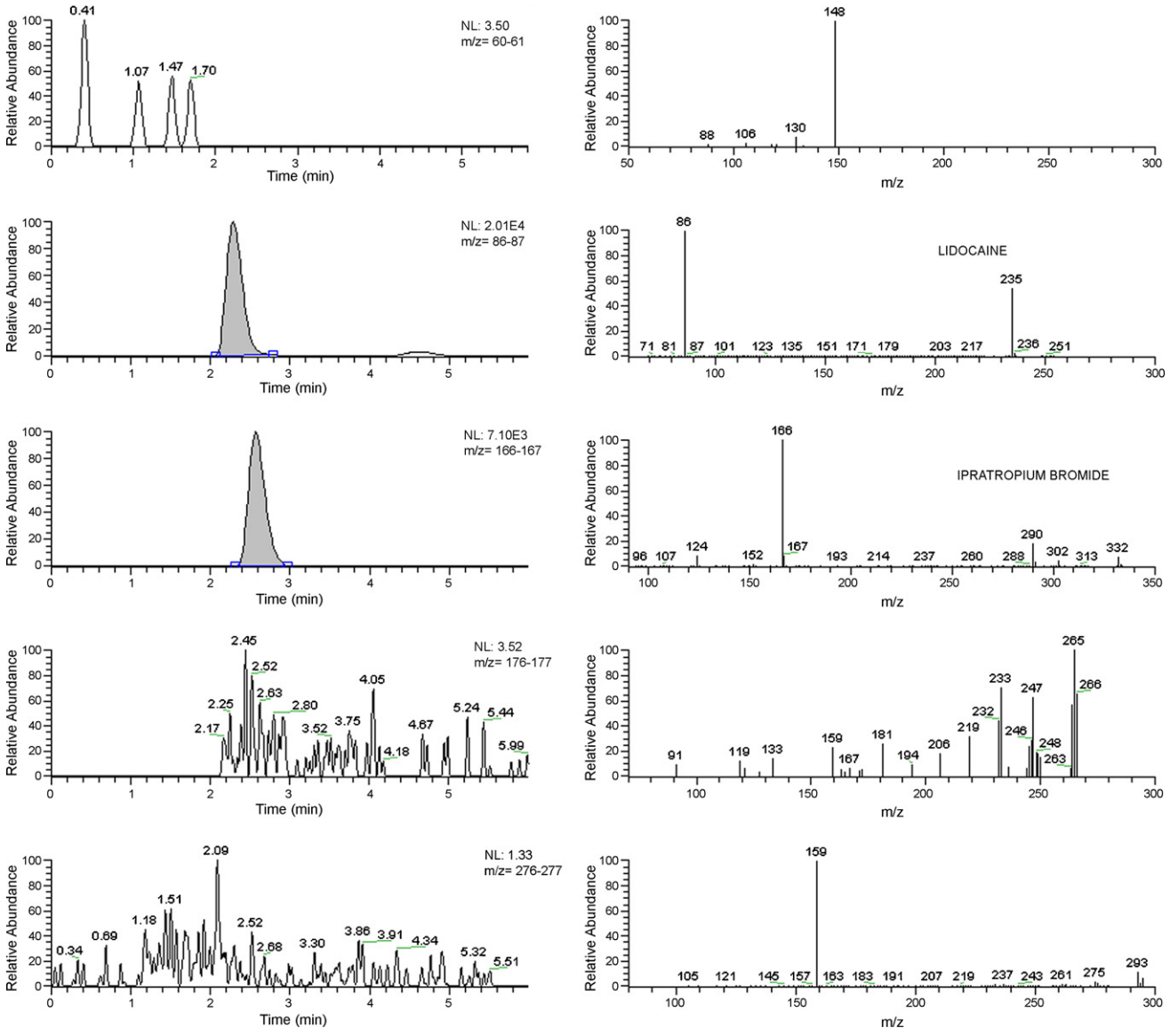


Fig. 4. Extracted ion chromatograms of equine blank serum sample after S.P.E. procedure.

The extraction recovery of each compounds is reported in Table 2.

3.1.4. Linearity

To test linearity, for each single analyte (mebenzonium iodide, embutramide, tetracaine) calibration curves were prepared by spiking different samples of serum to produce the calibration curve points equivalent with a range of 1–500 ng/ml.

Each sample contained also 50 ng/ml of lidocaine (I.S.) for embutramide and tetracaine analytes, while for mebenzonium iodide 50 ng/ml of ipratropium bromide (I.S.) was added.

Each calibration curve was three times repeated. In particular the concentration of analytes was calculated from the subsequent simple linear equations:

$$Y = 0.0074668 + 0.0005744X \quad \text{(Tetracaine hydrochloride)}$$

$$Y = 0.00688355 + 0.000574226X \quad \text{(Mebezonium iodide)}$$

$$Y = 0.00810455 + 0.000569561X \quad \text{(Embutramide)}$$

The calibration curves established for all compounds present correlation coefficients higher than 0.9981 as can be seen in Table 2. The software used for these calculations was Excalibur® 1.4.

This method exhibited a good linear response for concentrations ranging from 1 to 500 ng/ml.

Table 2
LOQ, LOD, recoveries and r^2

Analytes	LOQ (ng/ml)	LOD (ng/ml)	Extraction recovery (%)	Calibration curve (r^2)
Tetracaine hydrochloride	0.03	0.01	89	0.9988
Mebezonium iodide	5	1.5	78	0.9985
Embutramide	1	0.3	91	0.9981

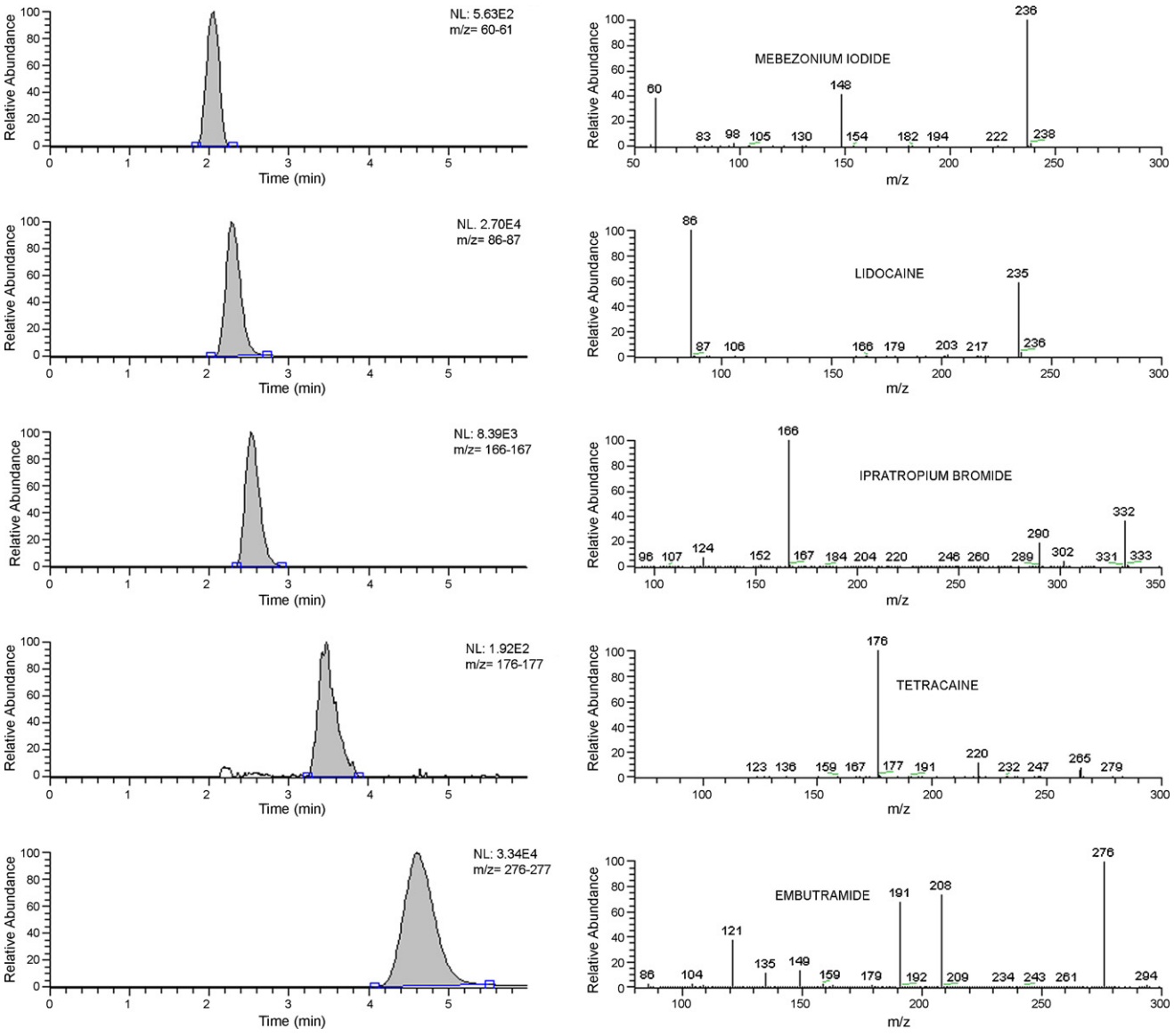


Fig. 5. Extracted ion chromatograms of a serum sample in a horse death case by a Tanax® administration.

3.1.5. Precision and accuracy

To access intra day precision three spiked serum samples at three concentration levels each (5, 50 and 500 ng/ml) were prepared and analysed. The procedure was repeated on seven different days to determine the inter day precision.

The range of repeatability and reproducibility coefficients were 5–8% and 7–14% (R.S.D.), respectively.

The obtained results were within the acceptance criteria for precision and accuracy which establish the deviation values that should be within ±15% of the actual values.

3.1.6. Stability study

Stability experiments were performed to evaluated the mebezonium, tetracaine and embutramide stability in stock solutions and in serum samples, conserved under different conditions simulating the same ones occurred during study sample analysis. In particular, the stock solutions, serum and final solid-phase extract samples were analysed at room and freeze temperature. The results obtained were well within the acceptable limit. Stock solutions of Tanax® active ingredients were stable at room temperature for 36 h, at 4 °C for 2 months and at −20 °C for 3 months. In the final solid-phase extract, Tanax® active ingredients at room temperature were stable for 30 h. The analytes spiked serum samples stored at 4 °C for long term stability experiments were found stable for at least for 1 month.

3.2. Application of the proposed method

A serum sample of a horse death cause of a suspected Tanax® administration was extracted and cleaned up according to the optimized method and analysed in order to prove the presence or not of single active ingredients of Tanax® speciality. Extracted ion chromatograms of the equine serum sample are shown in Fig. 5.

In this case, Tanax® analytes were detected with the studied method described above and it can be suggested the Tanax® administration to the animal. As is shown in Fig. 5 a qualitatively determination of each active ingredients of Tanax® speciality, as well as the internal standards added, is possible.

4. Conclusion

From literature data it is clear that Tanax® is an optimal drug for euthanasia in several animal species.

The most important problem derived from the use of this drug is represented by the identification of three active ingredients of Tanax® from biological fluids. In recent years the researchers have focused their attention on the identification of embutramide, mebenzonium iodide using for example GC–MS and HPLC–UV. In this paper, a rapid and very sensitive method, which allowed the simultaneous determination of all Tanax® active ingredients using LC–MS/MS has been described.

The method proposed, with a retention time of few minutes, was developed for a matrix (serum) after S.P.E. extraction.

In only 6 min the simultaneous detection of embutramide, mebenzonium iodide and tetracaine is possible.

The separation was carried out in isocratic conditions, that permitted to obtain a good reproducibility thanks even to the absence of the reconditioning times.

The method proposed was applied successfully to a real case of suspected Tanax® death horses which was brought to our notice.

It is possible to conclude that the above LC–MS/MS validated method can be highly useful to verify a suspected Tanax® administration to an animal and in a near future could revile a valid instrument for a rapid determination of Tanax® intoxication.

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