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Determination of embutramide in biological matrices by gas chromatography with nitrogen–phosphorus detection

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Abstract

Embutramide is a general anesthetic having a strong narcotic effect on the central nervous system where it paralyzes the brain center that controls respiration. It is a constituent of T61, a veterinary euthanasia drug. This paper describes a gas chromatographic procedure using nitrogen–phosphorus detection for the determination of embutramide in biological matrices. The drug and the internal standard (ambucetamide) are extracted with dichloromethane under alkaline conditions. The method is linear from 100 to 3000 ng/ml. The within-day and day-to-day coefficients of variation range from 5.1 to 5.7% and from 9.1 to 10.0%, respectively. The recovery is above 80% while the minimum detectable level under the conditions described is 40 ng/ml analyzing a 1-ml or a 1-g aliquot of a sample (blood or tissue). The method is also applied to different samples from dogs euthanized with T61.

1. Introduction

Embutramide (trade name: T61^{R}) has been marketed since 1961 as a veterinary euthanasia drug. It consists of a mixture of three compounds: embutramide or N-(ß,ß-diethyl-*m*-methoxyphenethyl)-4-hydroxybutyramide (200 mg/ml) (Fig. 1); mebezonium iodide or 4,4′-methylenebis(cyclohexyltrimethylammonium)diiodide (50 mg/ml); and tetracaine hydrochloride or *p*-butylaminobenzoyl-2-dimethylaminoethanol hydrochloride (5 mg/ml). The three compounds are dissolved in a mixture of dimethylformamide and water (6:4, v/v). Embutramide is a general anesthetic that has a strong narcotic effect on the central nervous system where it paralyzes the brain center that controls respiration. Mebezonium iodide exerts a curariform-like action paralyzing the striated muscles and rapidly inducing a circulatory collapse. Tetracaine hydrochloride is a local anesthetic which is

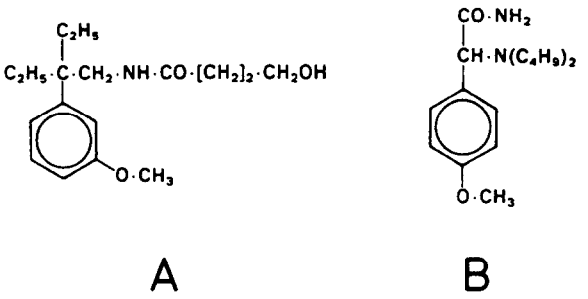

Fig. 1. Chemical structures of embutramide (A) and of the internal standard ambucetamide (B).

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added to reduce painful tissue reactions at the injection site [1]. The euthanasia results from central nervous system depression, hypoxia and circulatory collapse. The ultimate causes of death are hypoxia and respiration block due to depression of the vital centers and muscle paralysis.

T61 is injected preferably intravenously (0.3 ml/kg body weight) or intrapulmonary (1 ml/kg body weight) at a rate of 1 ml each 5 s. The use of T61 either by intravenous injection or oral ingestion has been reported in a number of deaths and suicide attempts [2–5]. In a number of patients who recovered from the neurological symptoms severe liver damage was observed. This hepatic damage was due to the dimethylformamide in the preparation [6,7].

Up to now only a very limited number of assays for the T61 constituents have been published, and little knowledge on the pharmacokinetics of these compounds is available. Thin-layer chromatography, followed by iodoplatinate spraying, allowed the separation and demonstration of embutramide and mebezonium iodide in biological matrices (blood, urine) [2,3]. Quantitative estimations were made by UV spectrophotometry either after elution of the compound from the silica gel (in the case of mebezonium iodide) or after extraction with diethyl ether (in the case of embutramide). It is clear that this procedure lacks the sensitivity and the specificity of the current chromatographic techniques. On the other hand a gas chromatographic–mass spectrometric (GC–MS) determination of embutramide in mammalian tissues has been described already in 1988 [8]. In this procedure large samples (20 g of tissue) and large volumes of extraction solvents (300 ml of acetonitrile and 100 ml of methylene chloride) were involved. In addition, extended sample clean-up (size-exclusion chromatography) was necessary before the final GC–MS measurement. A third procedure involves three extractions at different pH values and gas chromatographic analysis with flame-ionization detection (GC-FID) [5]. FID lacks specificity and often interferences are observed in the chromatograms, especially when originating from the complex matrices that are often encountered in toxicological analysis. In addition, in none of the above mentioned procedures internal standards were used.

We developed a simple and reliable procedure for the determination of embutramide in pharmacokinetic studies as well as in toxicological cases. The method is based on extraction of the matrix with dichloromethane followed by gas chromatographic analysis. The detection mode is nitrogen–phosphorus detection (GC-NPD) and ambucetamide [α-(dibutylamino)-4-methoxybenzeneacetamide] (Fig. 1) is used as an internal standard.

2. Experimental

2.1. Chemicals and reagents

Embutramide was a gift from Hoechst (Munich, Germany). T61 was commercially available and was manufactured by Hoechst Veterinär (Munich, Germany). $NaHCO_3$ was obtained from Merck (Darmstadt, Germany). All solvents were of analytical grade and were also from Merck. Ambucetamide was obtained from MCP Pharmaceuticals (Livingstone, UK). All derivatization reagents for gas chromatography were from Pierce Europe (Oud Beijerland, Netherlands).

2.2. Instrumentation

The experiments were carried out on a Hewlett-Packard (HP) 5890 Series II gas chromatograph (GC) equipped with a nitrogen–phosphorus detector. The GC was fitted with a 25 m × 0.25 mm I.D. Ultra-2 capillary column with a crosslinked 5% phenyl methyl silicone phase having a film thickness of 0.17 μm. The helium carrier gas was optimized at a back pressure of 95 kPa resulting in a linear velocity of 29.5 cm/s and a flow-rate of 0.67 ml/min at 150°C. The splitless injection port was maintained at 180°C while the septum purge-flow was switched off until 2 min after injection. The oven temperature was programmed from 150°C (2 min hold) to 280°C (5 min hold) at 10°C/min. The detector

temperature was set at 290°C. The chromatograms were recorded and the peaks were integrated with a Hewlett-Packard type 5840A GC terminal.

2.3. Stock solutions and standard solutions

The embutramide stock solution was prepared at 100 μg/ml in methanol while for ambucetamide the concentration was 20 μg/ml in dichloromethane. Both stock solutions were stored at −20°C. Appropriate dilutions with methanol (for embutramide) or with dichloromethane (for ambucetamide) yielded the standard solutions of both compounds. These standard solutions were also stored at −20°C.

2.4. Sample preparation

To a 1.0-ml volume of blood were added, 200 mg of $NaHCO_3$ to give a pH between 8.5 and 9.0, and 8 ml of dichloromethane containing 1 μg of ambucetamide as an internal standard. The extraction tubes were then placed on a rotary mixer for 15 min at a velocity of 42 rpm. After centrifugation for 10 min at 2000 *g* at room temperature the lower (organic) layer was transferred into a conical tube. The solvent was evaporated to dryness under a gentle stream of nitrogen, without heating. The residue was redissolved in methanol (two fractions of 0.6 and 0.4 ml, respectively) and the combined fractions were transferred into a 2-ml screw-capped vial. Again, the solvent was blown to dryness with a stream of nitrogen at room temperature. After addition of 50 μl of methanol the vials were vortex-mixed and a 2-μl aliquot was injected onto the GC system.

Tissue samples were homogenized with an Ultra Turrax mixer after dilution with water (1:1, w/w) and 2-g aliquots were extracted in exactly the same way as described for the blood samples.

If peak-area ratios were beyond the calibration range, samples were appropriately diluted with an isotonic NaCl solution and reanalyzed.

2.5. Calibration and quantitation

A standard curve for embutramide was prepared by adding embutramide to aliquots of a pool of drug free dog blood at concentrations of 100, 500, 1000, 2000 and 3000 ng/ml. These samples were then taken through the analytical procedure. The peak areas of embutramide relative to the I.S. were calculated and plotted versus the concentrations. The calibration curve was calculated using least squares regression. The concentration of embutramide in unknown samples was calculated from this regression line.

2.6. Reproducibility

Within-day reproducibility was evaluated by analyzing eight blood samples spiked with embutramide to obtain three different levels (100, 1000 and 3000 ng/ml). Day-to-day reproducibility was determined by analysis of seven replicate samples of each of the three levels (100, 1000 and 3000 ng/ml) on 7 consecutive days. Concentrations were determined using the calibration curve prepared on the day of analysis. From the data obtained within-day and day-to-day C.V. values were calculated.

2.7. Recovery

The recovery was evaluated by analyzing five blood samples of three different levels (100, 1000 and 3000 ng/ml) as described earlier, except that the internal standard was added just before injection. The control samples were prepared by mixing the same amounts of embutramide (100, 1000 and 3000 ng) and of the internal standard. The recovery was then determined by comparing the peak-area ratios from the spiked blood samples with those obtained from the injection of the two compounds dissolved directly in methanol.

2.8. Stability

Blood samples spiked with embutramide (100, 1000 and 3000 ng/ml) were stored for six months at 4°C. After that period these samples were

analyzed to evaluate the stability of this compound in blood.

2.9. Sensitivity

The limit of quantitation as well as the detection limit were estimated from the analysis of spiked blood samples (40 and 100 ng/ml, respectively).

3. Results and discussion

3.1. Optimization of the chromatographic conditions

Initially, degradation of embutramide was observed during the chromatographic process. By increasing the injector temperature from 150 to 250°C by increments of 10°C the peak area of the degradation product also increased. After evaluation of the peak area of the degradation product as well as of the peak shape of embutramide itself, the injector temperature was set at 180°C. Even the cold-on-column injection technique did not inhibit degradation. Different derivatization procedures such as silylation [with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) in pyridine, 30 min at 74°C], acetylation (with acetic acid anhydride, 210 min at 60°C), pentafluorobenzoylation [with pentafluorobenzylbromide in triethylamine and hexane (8:132, v/v)] and trifluoroacetylation [with trifluoroacetic acid anhydride in acetone–toluene (1:9, v/v) 60 min at 60°C] did not result in a substantial gain in stability of embutramide. Consequently, samples were injected underivatized and the splitless injection technique was preferred to lower the detection limit of the assay.

3.2. Sample preparation

Of the different extraction solvents tested (ethylacetate, mixtures of ethylacetate and *n*-hexane in different proportions, chloroform and dichloromethane) dichloromethane gave the highest recoveries and the least interferences.

For the extraction itself a rotary mixer was preferred above a vortex-mixing device because the latter caused emulsion formation resulting in a lower recovery.

3.3. Calibration

In whole blood samples the peak-area ratio (embutramide/I.S.) plot versus the concentration of added embutramide was linear ($r > 0.996$) over the range 100–3000 ng/ml.

3.4. Reproducibility

Table 1 shows the within-day and day-to-day reproducibility data of the embutramide analysis in whole blood samples at different levels.

3.5. Recovery

The total recoveries as obtained from the analysis of 5 samples with concentrations of 100, 1000 and 3000 ng/ml, yielded 81 ± 12%, 102 ± 4% and 86 ± 8%, respectively (mean ± S.D.).

3.6. Stability

The results of the stability study indicated a substantial degradation of embutramide under the conditions described. Analysis of the samples (100, 1000 and 3000 ng embutramide/ml) stored

Table 1
Accuracy and precision data of the embutramide analysis in whole blood

Level (ng/ml)	*n*	Found (mean ± S.D.) (ng/ml)	C.V. (%)
Within-day			
100	8	98 ± 5	5.7
1000	8	990 ± 54	5.5
3000	8	3189 ± 163	5.1
Day-to-day			
100	7	85 ± 9	10.0
1000	7	1114 ± 105	9.5
3000	7	3640 ± 331	9.1

for 6 months at 4°C resulted in the following concentrations: 88, 416 and 826 ng embutramide/ml, respectively. In samples stored at −20°C during six months no substantial degradation of embutramide was observed.

3.7. Sensitivity

Analyzing a 1-ml volume of whole blood the limit of quantitation was 100 ng/ml while the limit of detection was 40 ng/ml (signal-to-noise ratio of 3:1).

3.8. Application of the method

The method presented here was applied to the determination of embutramide in biological matrices (blood, heart, lung, liver and kidney) from four dogs (Table 2) euthanized with T61. The dogs were euthanized for serious illness (e.g. lung cancer, breathing problems and lymph carcinoma). T61 was administered intravenously at a rate of 1 ml each 5 s, as suggested by the manufacturer, and dogs 1, 2 and 3 died very quickly while dog number 4 died only after 5 min because it was not possible to inject successfully the total dose directly into the vein. Shortly after death, blood was taken from the heart and tissue samples were collected. The samples were kept in the freezer till analysis. For dog number 1, samples were collected only 14 h after death.

The samples were extracted and chromatographed as described in the Experimental section (see Figs. 2 and 3). Table 3 summarizes the concentrations found in the different matrices from the four dogs. As can be seen from Table 3 the embutramide levels in blood are very high in dog number 2 and 3 corresponding with the study of Braselton et al. [8]. The lower level found in dog number 1 is probably due to later collection of the sample (14 h after death) allowing degradation of the compound. For dog

Table 2
Characteristics of dogs euthanized with T61

Dog	Age (years)	Sex	Weight (kg)	Volume of T61 (ml)
1	10	M	45	14
2	12	M	10	3
3	6	F	32	10
4	12	F	8	2.4[a]

[a] Only part of the T61 was successfully injected into the vein.

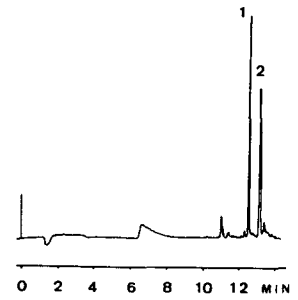

Fig. 2. Chromatogram of an extract of 1 ml of the appropriately 1 to 100 diluted blood sample of dog 1. Peaks: 1 = ambucetamide (internal standard), 2 = embutramide. Concentration of embutramide: 161 μg/ml.

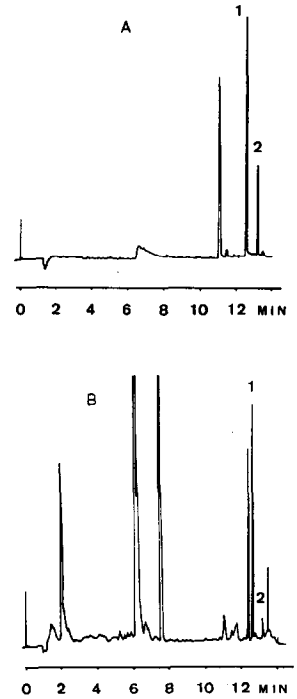

Fig. 3. (A) Chromatogram of an extract of 1 ml of a spiked blood sample (1 μg embutramide/ml). (B) Chromatogram of an extract of 1 g heart tissue. Concentration of embutramide: 0.36 μg/ml. Peaks: 1 = ambucetamide (internal standard); 2 = embutramide.

Table 3
Concentration of embutramide in biological matrices from dogs euthanized with T61

Matrix	Embutramide concentrations (μg/ml or μg/g)			
	Dog 1	Dog 2	Dog 3	Dog 4
Blood	161	671	776	69
Heart	0.79	501	3.37	0.36
Lung	0.97	105	0.28	5.91
Liver	BDL[a]	BQL	BQL	BQL
Kidney	NS	BDL	BDL	BQL

[a] BDL: below detection limit of 40 ng/g; BQL: below quantification limit of 100 ng/g; NS: no sample available.

number 4 the effectively injected (i.v.) dose was lower, as explained earlier, and the dog died only after five minutes possibly allowing redistribution and some metabolization of the embutramide.

The level of embutramide in heart and lung tissue was low in all dogs except in dog number 2. Only in dog number 4 (with the prolonged life after administration of the embutramide) traces of embutramide were detectable in the kidney sample, and the lung/heart embutramide level ratio was above 15. This indicates that the distribution of embutramide follows the blood flow to the different organs, i.e. to the lungs receiving close to 100% of the cardiac output and also to the kidneys which receive approximately 20% of the cardiac output.

As can be seen from the chromatograms shown in Figs. 2 and 3 there is no interference from the other constituents of T61 (mebezonium iodide and tetracaine) on the peak of embutramide and of the internal standard. This is probably due to a much lower recovery, a substantially lower concentration and a different chromatographic behavior.

4. Conclusions

The determination of embutramide in biological matrices is achieved by liquid–liquid extraction followed by gas chromatography and nitrogen–phosphorus detection. The method presented has been evaluated with respect to linearity, precision, recovery and sensitivity and is suitable for distribution studies of embutramide in euthanized dogs. This procedure can serve as a valuable alternative for the less sensitive and less specific UV spectrophotometric procedure as well as for the more sophisticated GC–MS procedure for the study of the pharmacokinetic behaviour of embutramide in various biological matrices.

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