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# Determination of tranquilisers and carazolol residues in animal tissue using high-performance liquid chromatography with electrochemical detection

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## ABSTRACT

A multi-residue method for the determination of tranquiliser residues in animal tissue is described. The procedure may be used to determine residues of the tranquilisers acepromazine, azaperone, chlorpromazine, haloperidol, propionylpromazine, xylazine, the metabolite of azaperone, azaperol, and the β-adrenoreceptor blocking agent carazolol. Existing methods of analysis for tranquilisers are based on ultraviolet and fluorescence detection and have been used for pig kidney analysis. Determination in this method was by high-performance liquid chromatography with electrochemical detection in the screen mode. The enhanced selectivity offered by the electrochemical detector allowed determination in liver extracts, which often give rise to more interferences on chromatographic traces when using conventional methods of detection. The method offers up to a ten-fold improvement in limits of determination over methods using ultraviolet and fluorescence detection. Recoveries and coefficients of variation have been determined in the range 2–25 μg/kg in pig kidney and liver. This electrochemical detection method has been used to measure residues in routine surveillance programmes.

## INTRODUCTION

Tranquilisers are administered to food-producing animals for a variety of reasons, which may result in residues in meat and meat products. The main categories of use are for sedation prior to handling or examining the animal, or to sedate an animal prior to transportation. It is the use of tranquilisers before transporting animals to slaughter which is most likely to result in residues entering the food chain, since they are administered only a few hours prior to this event and insufficient clearance time may have been allowed. Tranquilisers are used in this instance to minimise death and injury during transport, and to reduce stress. Stress is especially noticeable in pigs which have been bred to give lean meat, because they are more susceptible to this condition which gives rise to tough meat.

The β-adrenoreceptor blocking agents can be used to give effects similar to the neuroleptics. Amongst this class, carazolol is known to be used. The neuroleptics, or true tranquilisers act by blocking the dopamine receptor sites in the brain, which overload in times of stress. β-Adenoreceptor blocking agents act by slowing down the heart rate.

Some of the tranquilisers concentrate in the kidney and others in the liver. Kidney is the target organ for azaperone [1], azaperol [1] and xylazine [2] and liver the target organ for the phenothiazines [3,4]. Carazolol is reported in some places to concentrate in the liver [5], and in others, the kidney [4,6].

A recent review on the analysis of veterinary drug residues in edible animal products has been made by Shepherd [4] and covers methods available for the determination of these compounds in animal tissue. Early methods for the determination of individual or small groups of residues using liquid chromatography (LC) and gas chromatography (GC) have been summarised by Van Ginkel *et al.* [7].

Multi-residue methods for the determination of tranquilisers have been described by Keukens and

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Aerts [8] and by Van Ginkel *et al.* [7]. Both of these methods were evaluated in our laboratory, and best results were found using modifications of the extraction procedure used by Keukens and Aerts [8] and the high-performance liquid chromatographic (HPLC) prcedure used by Van Ginkel. *et al.* [7]. Both of these methods use UV detection for all compounds and additional fluorescence detection for azaperol, azaperone and carazolol.

Chlorpromazine has been detected in human plasma at therapeutic levels using electrochemical detection (ED) [9] and it was decided to investigate this method of detection as a potential confirmatory method of analysis.

## EXPERIMENTAL

Samples were prepared using a modification of the extraction and clean-up method described by Keukens and Aerts [8]. The HPLC method used was a modification of that described by Van Ginkel *et al.* [7].

### *Standards*

The analytical standards used were obtained as follows: acepromazine was a donation from Border Research (Dundee, UK); azaperol was purchased from Janssen Biotech (Olen, Netherlands), azaperone from Janssen Biochimica (Beerse, Belgium), carazolol from Boehringer Mannheim (Mannheim, Germany) and chlorpromazine, haloperidol, propiopromazine and xylazine from Sigma (Poole, UK).

### *Materials*

All solid chemicals, sulphuric acid and ammonia solution used in the procedure were AnalaR grade and obtained from BDH (Poole, UK). Water was obtained from an in-house Elga purification system, and other solvents were HPLC grade and obtained from Rathburn Chemicals (Walkerburn, UK). Bond-Elut $C_{18}$ solid-phase extraction cartridges were obtained from Jones Chromatography (Mid-Glamorgan, UK).

A 10% sodium chloride solution and a 0.01 *M* aqueous sulphuric acid solution were prepared. Acidic acetonitrile was prepared by the addition of 1 ml of 0.05 *M* sulphuric acid to 100 ml acetonitrile.

Amber vials (4 ml) and vial inserts (300 $\mu$l) were prepared by rinsing successively with concentrated ammonia solution, water and acetone. They were dried using a stream of nitrogen.

The HPLC eluent was prepared by mixing 0.77 g of ammonium acetate, 500 ml of acetonitrile and 500 ml of water. The solution was filtered through a 0.22-$\mu$m membrane filter and degassed before use using low pressure and an ultrasonic bath.

### *Procedure*

An excess of pig kidney was cut into small pieces and was homogenised in a 100-ml polypropylene centrifuge tube. About 5 g were accurately weighed out into a 50-ml polyethylene centrifuge tube. To the homogenate were added 10 ml of acetonitrile, and the tube was shaken. The sample was then mixed thoroughly for 30 s on a vortex-mixer and sonicated for 3 min in an ultrasonic bath. The mixing and sonicating processes were repeated, and the samples centrifuged at 10 000 *g* for 20 min.

After centrifugation, 7.5 ml of the supernatant were added to 40 ml of 10% sodium chloride solution in a 100-ml polypropylene tube. A Bond-Elut $C_{18}$ solid-phase extraction (SPE) cartridge was placed on a Vac-Elut evacuation chamber and activated successively with 5 ml of methanol and 5 ml of water. The extract was immediately added to a reservoir connected to the SPE cartridge and allowed to pass through slowly at a flow-rate of about 1 ml/min by applying an appropriate pressure. Care was taken to prevent the cartridge from drying out at this stage. When all of the sample had passed through the cartridge, it was flushed with 0.85 ml of 0.01 *M* sulphuric acid and dried with air. The analytes were eluted with 3.5 ml of acidic acetonitrile into a prepared 4-ml vial.

The vial was placed in a metal block heated to 50°C and the eluate was evaporated to dryness using nitrogen. The extract was taken up in 300 $\mu$l of 0.01 *M* sulphuric acid. The extract was mixed briefly using a vortex-mixer and 1 ml of hexane was added. The combined organic and aqueous layers were mixed for 30 a on a vortex-mixer and centrifuged at 2000 *g* for 5 min. The aqueous phase was transferred to a pre-treated 300-$\mu$l vial insert by inserting a Pasteur pipette through the organic layer and withdrawing the aqueous fraction. This extract was ready for analysis by HPLC.

*Chromatography*

The HPLC system was isocratic and consisted of an LKB 2150 pump, a Waters WISP 710B autoinjector, an in-line 0.2-μm filter, a Hypersil SAS $C_1$ guard column and a 25-cm Hypersil SAS $C_1$ (5 μm) analytical column. The flow-rate was maintained at 2.0 ml/min.

The electrochemical detector used was the ESA Model 5100A Coulochem detector with the Model 5010 analytical cell and the Model 5020 guard cell (supplied in the UK by Severn Analytical). The guard cell may be regarded as an accessory and was used to condition the mobile phase.

*Cyclic voltammetry*

Hydrodynamic voltammograms were constructed for each of the analytes. The first electrode of the analytical cell was held at 0.0 V and the potential at the second electrode was increased from 0.0 V in increments of 0.05 V. An injection of mixed tranquiliser standard was made onto the HPLC system after each adjustment. No response was seen up to +0.4 V. Peaks were seen above this potential at the retention times associated with each of the tranquiliser standards. No further significant response was observed after +0.7 V. The first electrode was then switched to +0.4 V and the second electrode increased from +0.4 V in steps of 0.05 V. The response of each analyte was measured at each potential. A hydrodynamic voltammogram is plotted in Fig. 1.

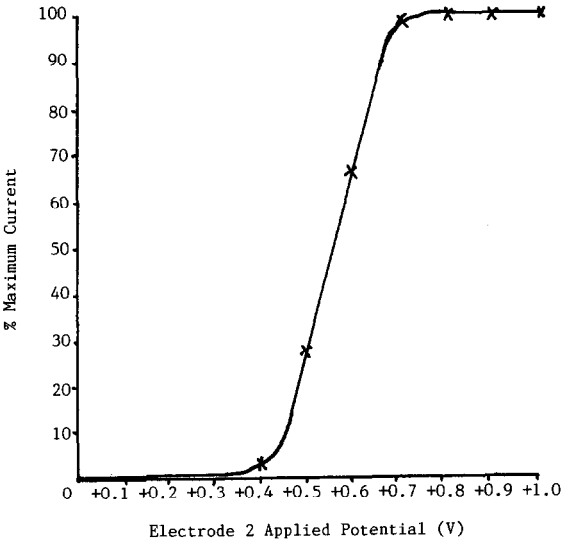


Fig. 1. Hydrodynamic voltammogram for tranquilisers. Electrode 1 was held at +0.4 V.

*Operation*

The guard cell was placed after the HPLC pump and before the injector. A potential of +0.75 V was applied to it in order to eliminate any response in the analytical cell which may be due to impurities in the mobile phase. The first electrode of the analytical cell was held at +0.4 V. The second electrode, from which the output was monitored, was set to +0.7 V. Using the combination of available electrodes in this manner is termed the sceen mode. The electrode 1 potential is at the foot of the current–voltage (*I–V*) curve for the analytes of interest. This is done to decrease background currents and to prevent unwanted peaks that result from eluents that oxidise at lower potentials than the analytes. The electrode 2 potential is set on the *I–V* curve plateau of the analyte with the highest oxidising potential. Compounds which oxidise at even higher potentials will not produce chromatographic peaks. This mode of operation increases the selectivity of the electrochemical detector.

Mobile phase was recirculated in the HPLC system by placing the waste outlet tubing into the reservoir. Recirculation is beneficial from both economic and environmental aspects, and has no detrimental effects when using this method. The mobile phase was regularly changed, especially if an increase in background current was observed in the analytical cell. Mobile phase was filtered through a 0.2-μm filter prior to use since the presence of small particles may block the porous graphite electrodes. An in-line filter was incorporated into the HPLC system after the injector as further protection for the electrodes. Sample extracts were filtered through 0.2-μm filters prior to analysis.

When an increase in back-pressure of more than 50 bar due to the analytical cell was noticed, it was isolated from the system and pumped through with 6 *M* nitric acid followed by water in order to clean it.

*Validation protocol*

Samples were processed in batches of eight. The spiking concentration and numbers of batches

TABLE I

RECOVERIES FROM PIGS' KIDNEY SPIKED WITH A MIXTURE OF COMPOUNDS AT 2 μg/kg

Values in parentheses are R.S.D.s (%).

| Compound | Recovery (mean ± S.D.) (%) | | |
|---|---|---|---|
| | Day 1 ($n=6$) | Day 2 ($n=6$) | Overall ($n=12$) |
| Azaperol | 75 ± 2.2 (3.0) | 81 ± 3.9 (4.9) | 78 ± 4.1 (5.3) |
| Azaperone | 74 ± 3.4 (4.6) | 86 ± 5.0 (5.9) | 80 ± 7.3 (9.1) |
| Carazolol | 76 ± 4.1 (5.4) | 70 ± 6.7 (9.5) | 73 ± 6.2 (8.4) |
| Xylazine | 68 ± 6.2 (9.3) | 56 ± 3.4 (6.0) | 62 ± 7.8 (12.5) |
| Haloperidol | 69 ± 3.2 (4.7) | 74 ± 2.2 (3.0) | 72 ± 3.8 (5.3) |
| Acepromazine | 70 ± 4.5 (6.3) | 87 ± 4.8 (5.6) | 78 ± 9.5 (12.2) |
| Propriopromazine | 70 ± 4.5 (6.3) | 88 ± 4.8 (10.7) | 78 ± 9.5 (17.3) |
| Chlorpromazine | 82 ± 2.7 (3.3) | 95 ± 7.4 (7.8) | 88 ± 8.8 (10.0) |

TABLE II

RECOVERIES FROM PIGS' KIDNEY SPIKED WITH A MIXTURE OF COMPOUNDS AT 10 μg/kg

Values in parentheses are R.S.D.s (%)

| Compound | Recovery (mean ± S.D.) (%) | | | |
|---|---|---|---|---|
| | Day 1 ($n=6$) | Day 2 ($n=6$) | Day 3 ($n=6$) | Overall ($n=18$) |
| Azaperol | 71 ± 4.3 (6) | 80 ± 2.4 (3) | 89 ± 1.9 (2) | 80 ± 8.0 (10) |
| Azaperone | 68 ± 4.2 (6) | 76 ± 1.0 (4) | 90 ± 5.0 (6) | 78 ± 10.1 (13) |
| Carazolol | 83 ± 6 (7) | 66 ± 2.9 (4) | 112 ± 14.1 (13) | 87 ± 21.3 (24) |
| Xylazine | 60 ± 3.7 (6) | 64 ± 1.7 (3) | 95 ± 8.9 (9) | 73 ± 16.9 (23) |
| Haloperidol | 68 ± 2.2 (3) | 64 ± 2.0 (3) | 94 ± 7.2 (8) | 75 ± 14.1 (19) |
| Acepromazine | 77 ± 2.3 (3) | 83 ± 4.4 (5) | 101 ± 6.5 (7) | 87 ± 11.4 (13) |
| Propriopromazine | 86 ± 3.8 (4) | 83 ± 5.0 (6) | 94 ± 6.5 (7) | 88 ± 6.5 (7) |
| Chlorpromazine | 81 ± 5.7 (7) | 83 ± 8.5 (10) | 100 ± 16.8 (17) | 88 ± 13.9 (16) |

TABLE III

RECOVERIES FROM PIGS' LIVER SPIKED WITH A MIXTURE OF STANDARDS AT 5 μg/kg

Values in parentheses are R.S.D.s (%).

| Compound | Recovery (mean ± S.D.) (%) | | | |
|---|---|---|---|---|
| | Day 1 ($n=6$) | Day 2 ($n=6$) | Day 3 ($n=6$) | Overall ($n=18$) |
| Azaperol | 86 ± 3.6 (4.2) | 91 ± 0.0 (0.0) | 62 ± 2.8 (4.5) | 80 ± 13.0 (16.4) |
| Azaperone | 72 ± 6.2 (8.7) | 77 ± 4.1 (5.3) | 52 ± 0.8 (1.6) | 67 ± 12.0 (17.9) |
| Carazolol | 95 ± 3.2 (3.4) | 89 ± 3.4 (3.9) | 72 ± 3.9 (5.4) | 85 ± 10.7 (12.6) |
| Xylazine | 73 ± 15.2 (2.0) | 78 ± 8.7 (11.2) | 46 ± 2.5 (5.4) | 66 ± 15.2 (23.1) |
| Haloperidol | 76 ± 2.2 (2.9) | 80 ± 11.6 (14.5) | 50 ± 4.9 (9.8) | 69 ± 15.2 (22.2) |
| Acepromazine | 79 ± 2.5 (3.2) | 81 ± 6.0 (7.4) | 58 ± 6.1 (10.6) | 72 ± 11.9 (16.5) |
| Propriopromazine | 80 ± 3.1 (3.8) | 79 ± 5.5 (7.0) | 61 ± 1.3 (2.1) | 74 ± 9.8 (13.3) |
| Chlorpromazine | 91 ± 2.5 (2.7) | 81 ± 5.3 (6.5) | 64 ± 3.1 (4.8) | 79 ± 12.0 (15.2) |

TABLE IV

RECOVERIES FROM PIGS' LIVER SPIKED WITH A MIXTURE OF STANDARDS AT 25 μg/kg

Values in parentheses are R.S.D.s (%).

| Compound | Recovery (mean ± S.D.) (%) | | | |
|---|---|---|---|---|
| | Day 1 ($n=6$) | Day 2 ($n=6$) | Day 3 ($n=6$) | Overall ($n=18$) |
| Azaperol | 81 ± 4.6 (5.7) | 82 ± 1.4 (1.7) | 90 ± 1.6 (1.8) | 84 ± 4.8 (5.7) |
| Azaperone | 78 ± 3.9 (5.1) | 80 ± 0.5 (0.6) | 85 ± 1.6 (1.9) | 81 ± 3.9 (4.8) |
| Carazolol | 78 ± 6.6 (8.4) | 84 ± 12.3 (14.7) | 82 ± 4.2 (5.1) | 81 ± 8.2 (10.2) |
| Xylazine | 70 ± 3.9 (5.6) | 70 ± 5.6 (8.0) | 82 ± 3.2 (3.9) | 74 ± 7.1 (9.6) |
| Haloperidol | 67 ± 2.8 (4.2) | 75 ± 1.6 (2.1) | 84 ± 3.1 (3.6) | 75 ± 7.7 (10.2) |
| Acepromazine | 82 ± 5.3 (6.5) | 90 ± 1.5 (1.6) | 88 ± 3.4 (3.8) | 87 ± 5.2 (5.9) |
| Propriopromazine | 82 ± 4.4 (5.4) | 92 ± 1.2 (1.3) | 95 ± 2.4 (2.5) | 90 ± 6.5 (7.2) |
| Chlorpromazine | 82 ± 3.4 (4.2) | 95 ± 1.6 (1.7) | 94 ± 1.6 (1.8) | 90 ± 6.7 (7.4) |

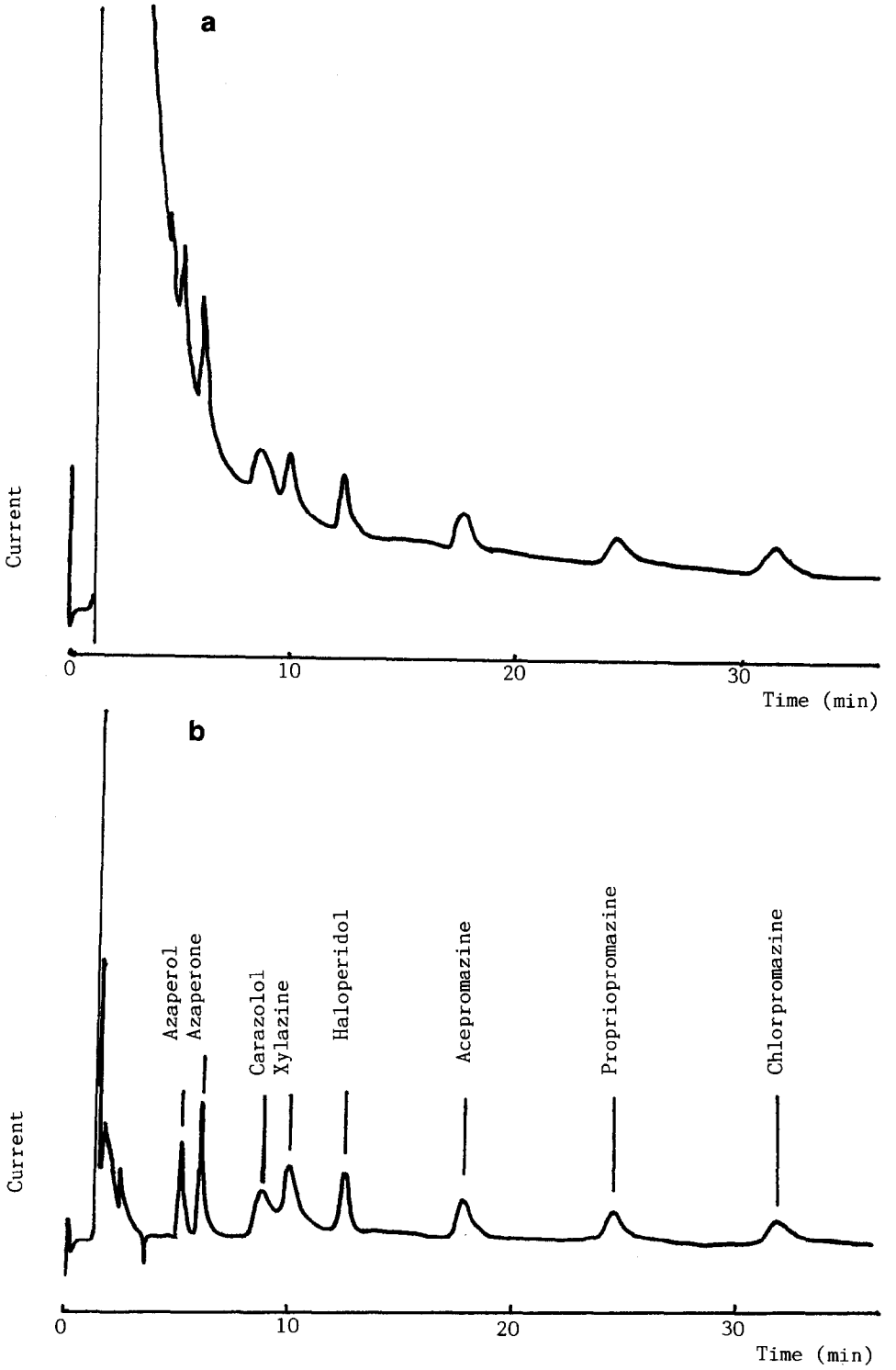


Fig. 2.

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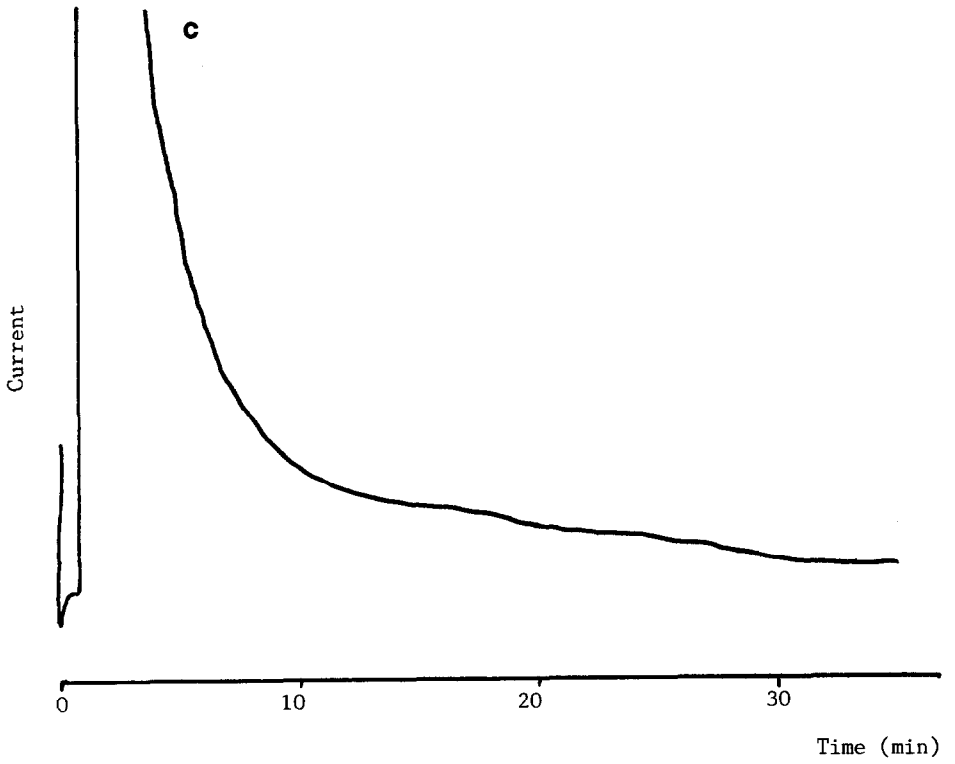


Fig. 2. ED chromatogram of (a) spiked tissue extract at 2 μg/kg, (b) mixed standard equivalent to 2 μg/kg and (c) blank tissue extract.

processed for each tissue type and concentration are detailed in Tables I–IV.

## RESULTS AND DISCUSSION

It was found that each of the tranquilisers gave a strong electrochemical response, and that each compound was oxidised at a similar potential (+0.4 to +0.7 V). This ED method has been adopted as a standard operating procedure in our laboratory and is routinely applied to the analysis of tranquilisers in meat samples.

The mean, standard deviation (S.D.) and relative standard deviation (R.S.D.) for the recovery of each standard in both tissue types are summarised in Tables I–IV. Typical chromatograms for a standard, spiked tissue extract and a blank tissue extract are shown in Fig. 2.

Limits of determination were found to be ten times lower than those found using UV detection.

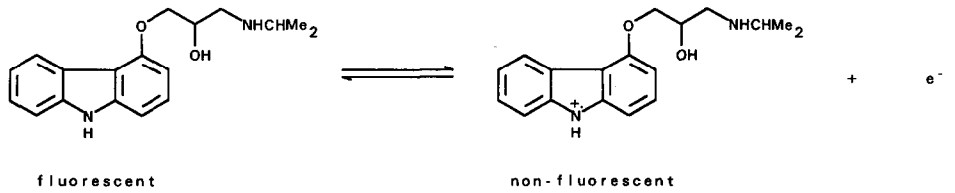


Fig. 3. Proposed reaction for the oxidation of carazolol. Me = Methyl.

TABLE V

COMPARISON OF LOWEST VALIDATION LIMITS FOR KIDNEY USING UV, FLUORESCENCE AND ELECTROCHEMICAL DETECTION

| Compound | Lowest validation limit (μg/kg) | | |
|---|---|---|---|
| | UV | Fluorescence | ED |
| Azaperone | 20 | 10 | 2 |
| Azaperol | 20 | 10 | 2 |
| Carazolol | 20 | 5 | 2 |
| Xylazine | 20 | N.A.[a] | 2 |
| Haloperidol | 20 | N.A. | 2 |
| Acepromazine | 20 | N.A. | 2 |
| Propriopromazine | 20 | N.A. | 2 |
| Chlorpromazine | 20 | N.A. | 2 |

[a] N.A. = Fluorescence detection not possible.

Significant improvements were also found for those compounds normally measured using fluorescence detection.

The electrochemical detector may be used in combination with other detectors. The peak associated with carazolol is not seen by fluorescence if the electrochemical detector is in series in front of the fluorescence detector. The peak is visible, however, if the detector positions are reversed. This suggests that the species produced when carazolol is oxidised is no longer fluorescent under the same conditions. This fact may be used to assist confirmation of samples screened as positive for carazolol, by analysing an extract twice with the detector positions reversed, or by using two detectors, one in front and one behind the electrochemical detector (Fig. 3).

## CONCLUSIONS

ED improves selectivity and enables the determiantion of tranquiliser residues in pig liver in addition to kidney samples. The method for the determination of tranquiliser residues presented in this paper offers up to ten-fold improvements in limits of determination when compared to existing methods (Table V).

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