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DETERMINATION OF RESIDUES OF CARAZOLOL AND A NUMBER OF TRANQUILLIZERS IN SWINE KIDNEY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND FLUORESCENCE DETECTION

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SUMMARY

A rapid and sensitive method has been set up for the determination of the beta-receptor blocker carazolol and the tranquillizers acepromazine, azaperone, chlorpromazine, haloperidol, propionylpromazine and xylazine in swine kidneys. The procedure involves extraction with acetonitrile, rapid sample clean-up with a Sep-Pak C_{18} cartridge and high-performance liquid chromatography with ultraviolet and fluorescence detection. The mean recoveries range from 93 to 101%, with the exception of xylazine (52%), and the coefficients of variation from 5.3 to 18.9% at a fortification level of 20 μg/kg. The limits of determination range from 0.3 μg/kg for carazolol to 1–10 μg/kg for the other drugs. The method has been tested in routine monitoring programmes.

INTRODUCTION

In veterinary practice, beta-receptor blocking agents and tranquillizers are used not only therapeutically but also to prevent stress during the transportation of swine and bulls from the farm to the slaughterhouse. Among the compounds claimed to be used, the blocking agent carazolol and the tranquillizers acepromazine, azaperone, chlorpromazine, propionylpromazine and xylazine have priority. Fig. 1 shows the molecular structures of the drugs investigated. It also includes azaperol, which is a major metabolite of azaperone[1], and haloperidol which is mainly used in human medicine.

At the start of this study no relevant data were available about which tranquillizers were used and the frequency of use in The Netherlands. In the F.R.G carazolol is used, but a withdrawal period of 3 days prior to transportation to the slaughterhouse has been established[2]. Still, recent data[3] from the F.R.G. indicated that an high percentage of swine kidneys analysed contained residues of carazolol in concentrations ranging from 0.4 to 10 μg/kg.

The purpose of the present study was to develop a routine multi-method for the determination of all compounds of interest in swine kidneys in the low μg/kg range.
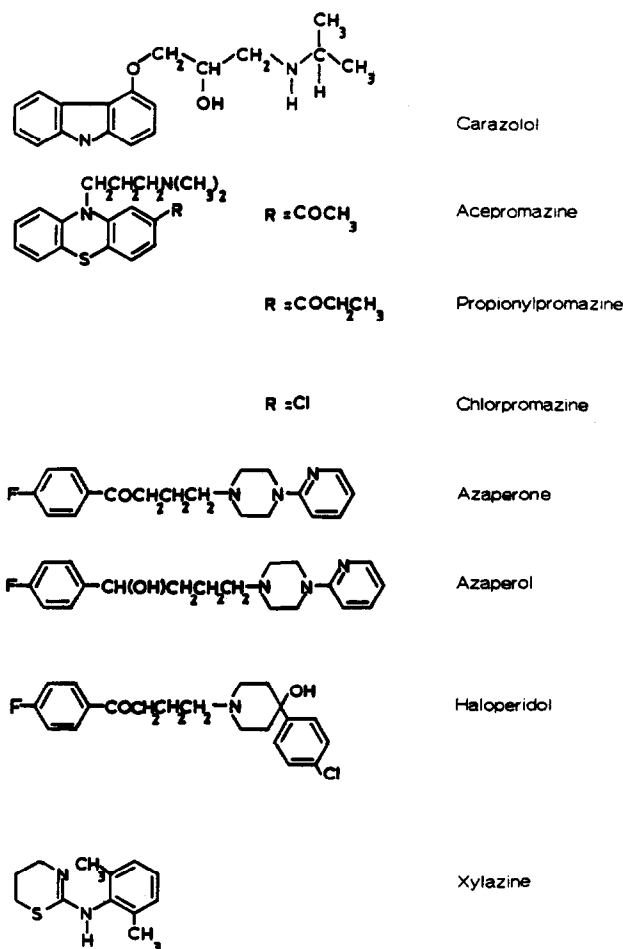
Fig. 1. Molecular structures of the drugs investigated.

Literature data indicated that the kidney was a good target-organ for monitoring the use of tranquillizers[3–6]. To our knowledge, up to now no quantitative multi-method for the determination of all the compounds mentioned has been described in literature. Among the published methods that are mostly applicable only to a single or a few compounds, are a fluorimetric method for carazolol[6], the thin-layer chromatographic (TLC) determination of azaperone and azaperol[1,4], of propionylpromazine[5] and of azaperone, propionylpromazine and carazolol[7], the gaschromatographic (GC) determination of acepromazine, propionylpromazine, chlorpromazine and xylazine[8] and of azaperone[5], an high-performance liquid chromatographic (HPLC) determination of carazolol[2,3] and a radioimmunoassay (RIA) method for carazolol[9]. An HPLC method that was used for the determination of all the relevant tranquillizer drugs except carazolol described by Etter et al.[10] showed low recoveries (<60%) and an unsatisfactory precision as indicated by high coefficients of variation.

We developed a simple and rapid method to determine residues of all beta-blocker and tranquillizer compounds mentioned in swine kidneys, using a combination of off-line solid phase extraction clean-up and HPLC separation with UV and fluorescence detection.

MATERIALS AND METHODS

Caution: the compounds investigated are light sensitive; their analysis should be under artificial yellow light using amber glassware.

Standards

Acepromazine, azaperone, chlorpromazine, propionylpromazine and xylazine were a generous gift from the Technical University Berlin (F.R.G.) and azaperol, carazolol and haloperidol from the National Institute of Public Health (Bilthoven, The Netherlands). Stock solutions of 100 μg drug per ml methanol were prepared in amber glassware. These solutions proved to be stable for 6 months when stored in the dark at 6°C.

Working standard solutions contained 100 ng drug per ml 0.01 M sulphuric acid except carazolol (50 ng/ml) and xylazine (200 ng/ml). These solutions were stable for at least 1 week when stored in the dark.

Reagents

All chemicals were of analytical grade (Merck, Darmstadt, F.R.G.) except where stated otherwise. A 10% aqueous sodium chloride solution and a 0.01 M aqueous sulphuric acid solution were prepared. Acidic acetonitrile was prepared by addition of 1 ml 0.05 M sulphuric acid to 100 ml acetonitrile.

The HPLC eluent was prepared by mixing 2.46 g anhydrous sodium acetate, 450 ml water and 550 ml acetonitrile Uvasol. The pH was adjusted to 6.5 with acetic acid. The eluent was filtered before use. The Sep-Pak C_{18} cartridges were obtained from Millipore (Milford, MA, U.S.A.).

Instrumentation

The HPLC system consisted of an automatic sampler Model WISP 710B (Millipore), a solvent-delivery system Model 6000A (Millipore), an UV–VIS detector Model Spectroflow 783 (Applied Biosystems, Foster City, CA, U.S.A.), a fluorescence detector Model 1046A (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a double pen recorder. The analytical column was a 300 mm × 3.9 mm I.D. column packed with Bondapak C_{18} (Millipore) and the guard column (10 mm × 2.1 mm) contained Bondapak C_{18}, 37–50 μm.

The eluent flow-rate was 1.2 ml/min. UV detection was performed at 240 nm with a range setting of 0.002 a.u.f.s. and a risetime of 5 s. Fluorimetric detection was performed at an excitation wavelength of 246 nm and an emission wavelength of 351 nm. The gain setting was 12, the risetime 4 s, the excitation slit width 2 mm and the emission slit width 4 mm. No cut-off filter was used. The recorder speed was 5 mm/min and the mV-setting for both signals was 10.

Analytical procedure

Excessive fat was removed from the kidney sample, which was then cut into small pieces and homogenized with a kitchen grinder. A 5-g amount of homogenized sample was accurately weighed in a polypropylene centrifuge-tube and 20 ml acetonitrile were added with continuous gentle mixing. After closing the tube, the sample was vigorously mixed on a vibromixer for 30 s (1500 rpm). The tube was placed in an ultrasonic bath for 2 min and then centrifuged at 4000 g for 5 min.

A Sep-Pak C_{18} cartridge was activated successively with 5 ml methanol and 5 ml water. To 7.5-ml volume of the sample extract were added 40 ml of 10% sodium chloride solution and mixed. This solution was gently pressed through the activated cartridge with a disposable syringe. The cartridge was flushed with 1 ml of 0.01 M sulphuric acid and next with 2 ml air. The compounds were eluted with 2 ml acidic acetonitrile and the eluate was collected in a calibrated tube which had been rinsed before use with concentrated ammonia, water and acetone and then dried under a stream of nitrogen.

The eluate was evaporated to 300 μl with a gentle stream of nitrogen at 70°C. After gentle mixing, 1 ml of *n*-hexane was immediately added and mixed for 30 s on a vibromixer. The phases were separated by centrifugation (2000 g) and the lower aqueous phase isolated. A 50-μl volume of the latter were injected into the HPLC system. A flow diagram of the analytical procedure is shown in Fig. 2.
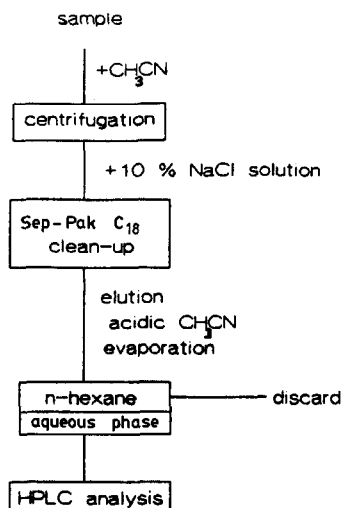

Fig. 2. Flow diagram of the analytical procedure.

RESULTS AND DISCUSSION

Chromatography

The objective was to use isocratic HPLC. An eluent mixture of acetonitrile and an acidic acetate buffer as described by Etter *et al.*[10] in combination with a column packed with a cyanopropyl bonded phase gave good separation for all compounds.

However, the peak heights and retention times of the injected standard solutions were not reproducible. Furthermore the kidney matrix severely influenced the retention times, possibly due to small variations in the pH of the final sample extract.

Therefore the use of C_{18} bonded phases was investigated. Here, the retention characteristics of the tranquillizers were strongly influenced by the endcapping of the column material, especially when acidic eluents were used. Only column materials where the silanol groups are sufficiently endcapped as for instance Bondapak (Millipore) and Supelcosil (Supelco, Bellefonte, PA, U.S.A.) were applicable. Other packing materials such as Chromspher C_{18} (Chrompack, Middelburg, The Netherlands) and LiChrosorb (Merck) showed strong adsorption effects. The promazines were not eluted from these columns when an acidic eluent was used. This was probably caused by strong polar interactions of the protonated basic drugs with available silanol groups.

The most reliable and reproducible results were achieved with a Bondapak C_{18} column with an acidic eluent. It was also essential to use Bondapak material for the guard column. The other materials tested caused strong adsorption of the compounds.

The retention behaviours of the compounds were strongly dependent upon the concentration of the acetate buffer and the pH of the eluent. The retention of all compounds decreased with increasing salt concentrations and at lower pH values, as expected from the basic properties of the drugs. The relationship between the retention time and acetate buffer concentration is shown in Fig. 3 for carazolol, azaperone and propionylpromazine. At a buffer concentration below 0.01 M the promazine and xylazine showed strong adsorption resulting in very asymmetric peaks. When the acetate concentration was increased to 0.1 M complete resolution was not obtained
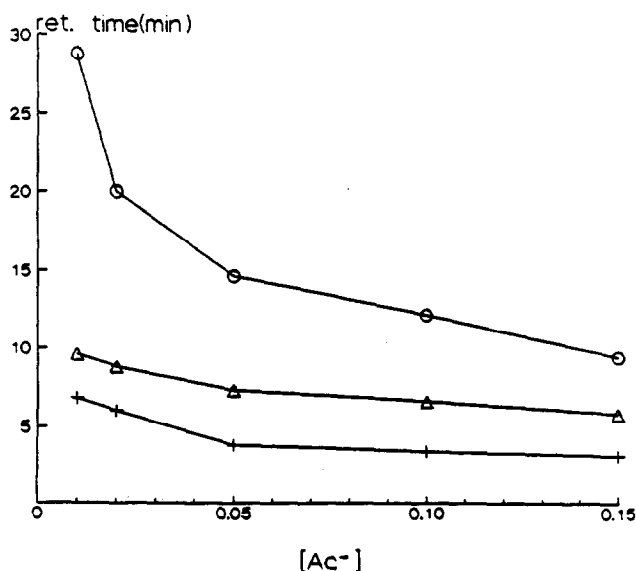

Fig. 3. Relationship between the acetate (Ac) buffer concentration and the retention time for carazolol (+), azaperone ($\triangle$) and propionylpromazine ($\bigcirc$). The eluent was acetonitrile–sodium acetate buffer (45:55, v/v) at pH 7, flow-rate 1.5 ml/min. The column was a Bondapak C_{18} (300 mm × 3.9 mm).

between carazolol and xylazine, even when the percentage of acetonitrile in the eluent was lowered from 55 to 40%. A buffer concentration of 0.03 M gave optimum results.

By varying the pH of the mobile phase, the separation of the compounds was further optimized. Depending upon the efficiency of the column used, the pH is set between 6.5 and 7; pH values below 6.5 should not be used. Fig. 4 shows the influence of lowering the pH of the mobile phase from 6.4 to 6.0. Excessive matrix interferences at the retention time of azaperone are observed at these lower pH values.
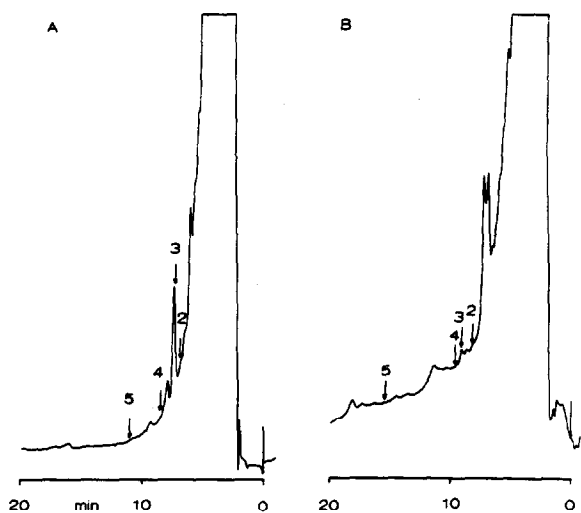

Fig. 4. HPLC chromatograms of a blank kidney sample analysed with an eluent at (A) pH 6.0 and (B) pH 6.4. The experimental conditions were as described in Materials and methods. Peaks: 2 = xylazine; 3 = azaperone; 4 = haloperidol; 5 = acepromazine.

Detection

All the drugs under investigation were detected with UV detection. Absorbance maxima ranged between 240 and 260 nm with the exception of xylazine which showed an optimum below 225 nm. As a compromise, 240 nm was selected as the detection wavelength.

Carazolol can also be determined with fluorescence detection[2,6]. Under the HPLC conditions described above, carazolol showed an excitation spectrum (emission wavelength 351 nm)[6] with a flat optimum between 230 and 246 nm (Fig. 5). This is not in line with the 283 nm observed by Rudolph and Steinhart[2]. An excitation wavelength of 246 nm was chosen because at lower wavelengths the signal-to-noise ratio decreased. In contrast with the findings of Engelsma and Simons[6], a fluorescence signal was also observed for azaperone and azaperol. The latter compound was not fully separated from carazolol. However, the ratio of fluorescence and UV responses for the two drugs can be used to differentiate in case of doubt. Under the experimental conditions used, this ratio was 14.6 for carazolol and 6.4 for azaperol.

Furthermore baseline separation of carazolol and azaperol is possible using an eluent with an acetate buffer concentration of 0.1 M and a pH 7.

Fig. 5. Fluorescence excitation spectrum for carazolol (———) and the eluent (·········) taken by a stop flow scan at emission wavelength 351 nm; scan speed 1.5 nm/s; paper advance 1 mm/s. The HPLC conditions as described in Materials and methods.

The fluorescence response of azaperone and azaperol allows a very selective determination of these compounds without matrix interferences.

Extraction and sample clean-up

In our hands a number of methods described in the literature for extraction and sample clean-up for some tranquillizers in kidney samples[1,5,7] yielded low and very irreproducible recoveries. The method of Etter et al.[10] was not investigated owing to the described irreproducible results.

Therefore a number of methods already in use within our laboratory for the extraction and clean-up of compounds with comparable physicochemical properties as for the substances under investigation were tried on kidney samples spiked with the tranquillizer drugs. First, a combination of an aqueous extraction and an Extrelut® cartridge clean-up was tested because tranquillizers were eluted quantitatively from the cartridge with dichloromethane. This procedure was similar to the one used for the determination of chloramphenicol in meat[11]. The final residue was dissolved in 0.01 *M* sulphuric acid and extracted with light petroleum (b.p. 40–60°C), as described by Etter et al.[10] to remove residual fatty material. The recovery percentages were quite acceptable (over 60%) for carazolol, xylazine and azaperone but very low (less than 30%) for haloperidol and the three promazines. In the literature low recoveries for tranquillizers are often attributed to interactions with active sites on glassware surfaces[2,10]. However when using polypropylene materials we observed no significant improvement. Spiking of haloperidol and the promazines at different stages of the sample clean-up showed that these drugs were not completely extracted from kidney samples with water. Therefore, a different approach using a non-aqueous extraction was chosen. Since organic solvents are not compatible with Extrelut, sample enrichment and clean-up was performed on Sep-Pak C_{18} cartridges.

With acetonitrile as the extractant, an aliquot of the organic extract was diluted in water to increase the polarity of the solution in order to retain the compounds of interest. However, it was impossible to retain the polar compounds carazolol, xylazine and azaperol in this way. When water was replaced by a 10% sodium chloride solution, all drugs, except xylazine, were however fully retained on the cartridge.

The use of the sodium chloride solution made it then necessary to flush the

cartridge with water before the actual elution to remove salts. No tranquillizers were lost by this step. At this stage of the study the recoveries obtained were generally acceptably high, but for the promazines not very reproducible. Also, the fluorescence chromatograms showed an interfering peak near carazolol. This interference was caused by the light petroleum used. Replacement by *n*-hexane did not lower the recoveries and no matrix interferences were observed. Typical fluorescence chromatograms for carazolol after extraction with light petroleum and *n*-hexane are given in Fig. 6.
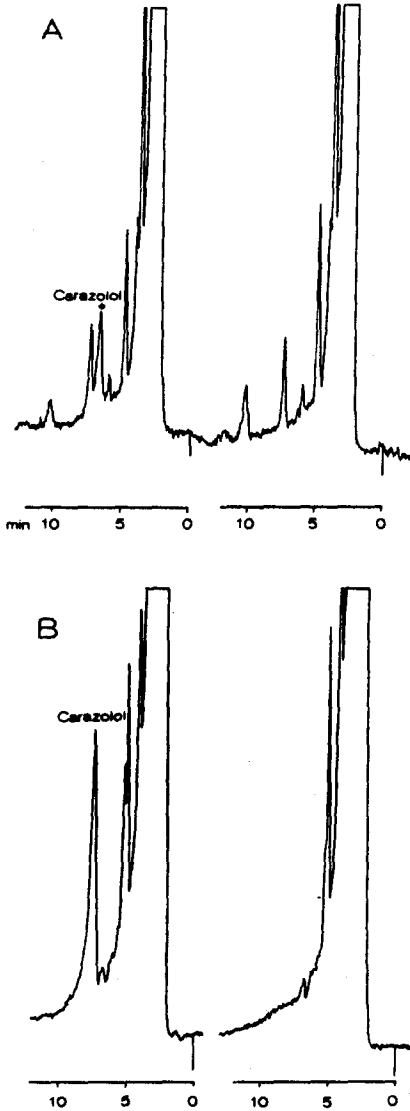

Fig. 6. Fluorescence chromatograms for (A) a blank and carazolol-spiked (1 μg/kg) kidney sample extracted with light petroleum and (B) a blank and a real kidney sample with carazolol (2 μg/kg) extracted with *n*-hexane. The HPLC conditions were as in Materials and methods.

Initially the irreproducible results for the promazines were attributed to losses during evaporation to dryness of the cartridge eluate. To solve this problem, the cartridge eluate was evaporated to a volume of 300 μl, mainly water. However, the solution remaining after evaporation had to be acidic, at least 0.01 M, to prevent substantial losses ($>80\%$) during the partition with n-hexane. The most simple way to obtain an acidic solution after evaporation was to flush the cartridge after sample enrichment with 0.01 M sulphuric acid instead of water.

This change resulted in more reproducible recoveries but still occasionally low recoveries for chlorpromazine were obtained. When however the hexane partition was performed immediately after evaporation to 300 μl, no losses occurred and the recoveries were generally about 90%, except for xylazine (50%). A possible explanation for the considerable improvement in reproducibility and recovery might be that the three (apolar) promazines are adsorbed to residual fats when the final aqueous extract cools down and are then co-extracted with the fat particles into the hexane phase. Immediate partition of the final (still slightly warm) extract prevents this. The low recovery for xylazine is found because the enrichment of this compound on the Sep-Pak C_{18} is critical. When 5 ml instead of 7.5 ml of the crude acetonitrile extract were applied to the cartridge, the recovery for xylazine was also 90%. Since this would mean an higher limit of detection for the other drugs, this change was not incorporated in the method.

The final procedure, using an acetonitrile extraction, dilution in saline solution, enrichment on a Sep-Pak C_{18} cartridge with mild acidic removal of salts, elution of the drugs with acidic acetonitrile and partition with n-hexane showed high recoveries, clean chromatograms and a good precision as shown in the analytical method characteristics below. Typical UV chromatograms of kidney samples are shown in Fig. 7.

Analytical method characteristics and applications

Recoveries and precision. Recovery experiments were carried out on different days by addition of 100 μl of a standard solution in water to the homogenized kidney samples. Carazolol was added at a level of 10 μg/kg, xylazine at a level of 40 μg/kg and all other compounds at a level of 20 μg/kg. The results are shown in Table I.

For azaperol, the azaperone metabolite, mean recoveries of more than 90% were obtained, but it was not routinely included in the standard spike solution. Xylazine showed the highest coefficient of variation caused by the critical sample enrichment on the Sep-Pak cartridge. To improve further the precision, the addition of an internal standard to the final HPLC extract can be recommended, mainly because the calibration of the tubes used in the final evaporation step is not very reliable.

In that case haloperidol can be used as an internal standard. This drug is normally used for human purposes only and is separated from the other tranquillizers in this system.

The stability of spiked kidney samples was tested by storing them at $-20°C$ for 1 month. No loss of recovery was observed. Recoveries are linear over the range of 0.01 to 1 mg/kg.

Limit of determination. Several hundreds of blank kidney samples were analysed with the method proposed. No interferences or significant noise were observed in the UV chromatograms. This made it difficult to carry out a calculation of the limit of
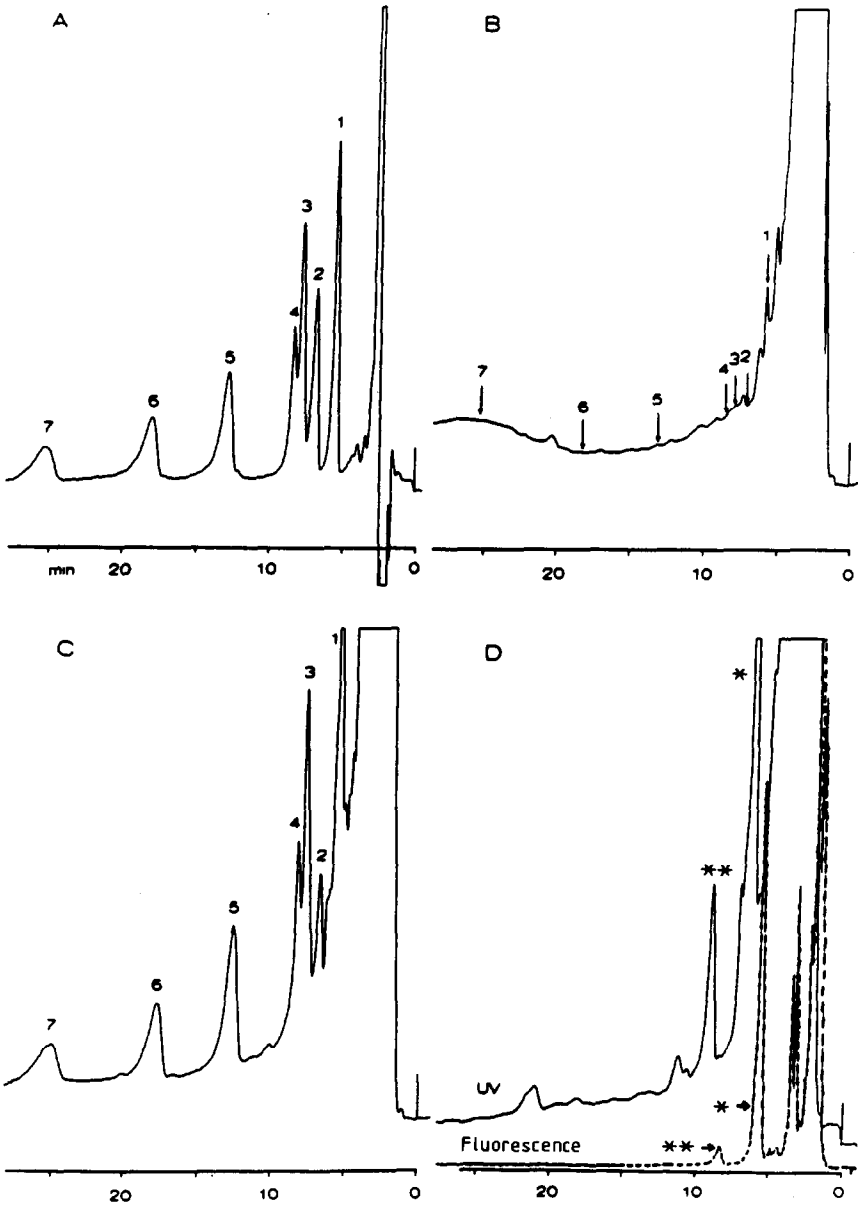
Fig. 7. HPLC chromatograms with UV detection of (A) a mixed standard solution containing 0.2 μg/ml of the drugs except carazolol (0.1 μg/ml) and xylazine (0.4 μg/ml); (B) a blank kidney sample; (C) a blank kidney sample spiked with 50 μg/kg of the tranquillizers except carazolol (25 μg/kg) and xylazine (100 μg/kg) and (D) a practice kidney sample from an azaperone-dosed swine. The HPLC conditions were as described in Materials and methods. Peak: 1 = carazolol; 2 = xylazine; 3 = azaperone; 4 = haloperidol; 5 = acepromazine; 6 = propionylpromazine; 7 = chlorpromazine; (D) only, * = azaperol; ** = azaperone.

TABLE I

RECOVERIES OF TRANQUILLIZERS AND CARAZOLOL IN SWINE KIDNEY SAMPLES ($n = 10$)

No.	Name	Fortification ($\mu g/kg$)	Mean recovery (%)	Coefficient of variation (%)
1	Carazolol	10	99	5.3
2	Xylazine	40	52	18.9
3	Azaperone	20	99	8.8
4	Haloperidol	20	95	7.6
5	Acepromazine	20	101	8.2
6	Propionylpromazine	20	95	6.7
7	Chlorpromazine	20	93	13.4

detection. This calculation should be based on the mean blank signal plus three times the standard deviation of the blank[12]. Therefore, as an estimation of the practical limit of determination, a peak height of 5 mm for the UV signal at a range of 0.001 a.u.f.s. and 10 mm for the fluorescence signal, in both cases representing more than 10 times the mean noise, was chosen. The results are shown in Table II.

Applicability and interferences. To test further the applicability of the method, a positive kidney sample from a carazolol-dosed swine was analysed as a reference sample in a number of monitoring program sample series. The mean level found was 2.0 $\mu g/kg$ ($n = 14$) with a coefficient of variation of 14.4%. This gives a good indication of the within-lab reproducibility of the carazolol determination. The somewhat higher C.V. compared with the 10-$\mu g/kg$ spike results is probably caused by the lower concentration and inhomogeneity of real kidney samples. The C.V. found easily meets the requirements stated by the European Economic Community for the repeatability of methods to be used for detecting residues of groups of veterinary drugs[13]. A value of 20% is considered to be acceptable in the concentration range 2–10 $\mu g/kg$.

More than 40 veterinary drugs and other (human) tranquillizers were injected into the HPLC system to check for interferences. Only perphenazine and promazine

TABLE II

LIMIT OF DETERMINATION OF TRANQUILLIZERS IN SWINE KIDNEYS

Results expressed in $\mu g/kg$.

No.	Name	Limit of determination[a]
1	Carazolol	0.3[b]
2	Xylazine	4
3	Azaperone	1
4	Haloperidol	2
5	Acepromazine	2
6	Propionylpromazine	4
7	Chlorpromazine	6
8	Azaperol	2[b]

[a] Defined as described in the text.
[b] Based on fluorescence detection.

showed UV peaks with retention times near propionylpromazine. However, these drugs are not expected in animal tissues and HPLC separation seems possible after optimization.

The method was tested in routine monitoring programmes. Over 1000 samples were analysed. With this method, 30 samples could be analysed per day without technical problems. Although the method was originally developed for kidney samples, it proved also applicable to injection sites, plasma and liver samples. Preliminary drug excretion studies have recently been carried out. The results will be reported elsewhere.
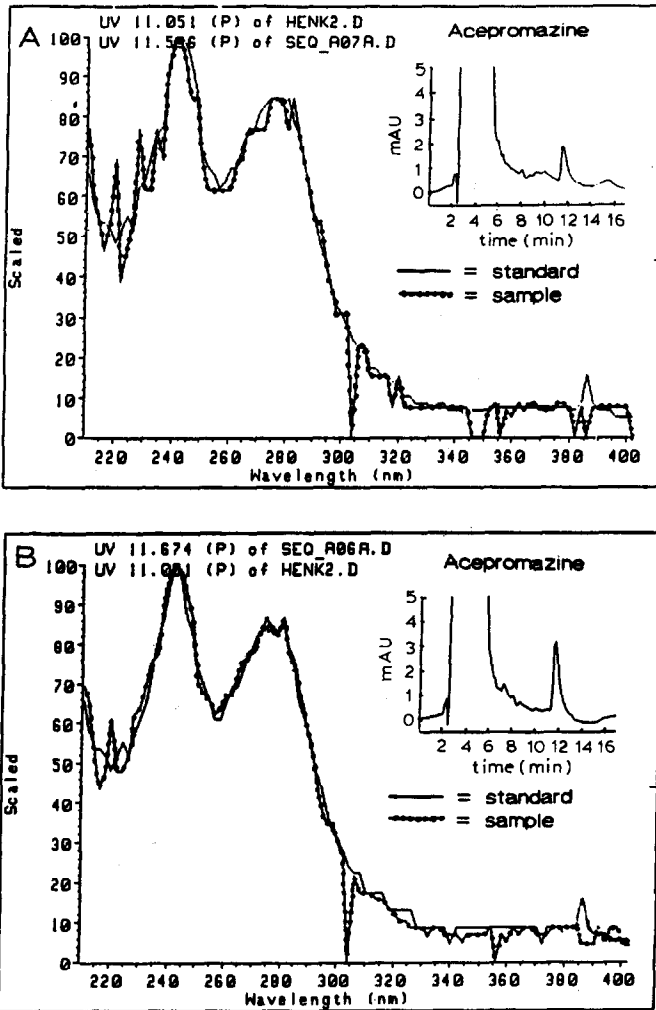

Fig. 8. HPLC chromatograms and co-plotted standard and sample spectra for two practice samples containing 40 and 80 μg/kg acepromazine. The HPLC conditions were as described in Materials and methods; an HP 1040 M UV–VIS detector was used.

Confirmation. When a diode array UV–VIS detector was used (Hewlett-Packard 1040 M), confirmation by co-plotting of UV spectra of the sample peak and standards was possible above a level of about 50 μg/kg. Chromatograms and spectra of two samples containing 40 and 80 μg/kg acepromazine, respectively, are shown in Fig. 8.

CONCLUSIONS

With the method developed, the tranquillizers acepromazine, azaperone (including its metabolite azaperol), chlorpromazine, haloperidol, propionylpromazine, xylazine and the beta-blocking agent carazolol can be determined in swine kidneys with a limit of determination ranging from below 1 μg/kg for carazolol to 1–10 μg/kg for the other drugs.

The analytical recoveries are high (93–101%) except for xylazine (52%) and the precision is excellent (coefficients of variation between 5.3 and 18.9%). The method was tested successfully in routine monitoring programmes. Although developed for kidney samples, the method can also be used for meat, plasma and liver samples. Further research is planned on the confirmation of positive results with diode-array UV–VIS and mass spectrometric detection.

ACKNOWLEDGEMENTS

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