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Note

Gas chromatographic assay of atropine and phenobarbital in pharmaceutical preparations containing Valeriana liquid extract

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Currently there are thousands of commercially available dosage forms with both antispasmodic and sedative actions. Some of them contain phenobarbital and a small quantity of atropine in a relatively large amount of Valeriana extract.

The aim of this work was to develop sensitive and reproducible methods for quantification of atropine and phenobarbital in the presence of valepotriates —the main active substances in Valeriana— and other natural products.

Previous procedures of assaying dosage forms for atropine and phenobarbital relied largely on titrimetry. These methods lack specificity and the required sensitivity in this case. Spectrophotometric measurements cannot be carried out, even after a lot of separation procedures, because of interfering coloured materials and other substances. Furthermore, atropine has little absorption in the UV region. On the basis of these facts, gas chromatography (GC) was chosen for solving the problem.

Both the British Pharmacopoeia 1980 (ref. 1) and the United States Pharmacopoeia XX[2] recommend GC for quantification of atropine in pharmaceutical formulations. Determination of the extracted atropine base is carried out by the official GC procedures. Neither of these methods is applicable in the presence of Valeriana liquid extract because of co-extracted interfering substances. With these considerations in mind the most likely approach was the GC analysis of one of the hydrolysis products of atropine. Quercia *et al.*[3] reported a GC method for assaying atropine in the form of tropine in pharmaceutical preparations, and Grabowski *et al.*[4] developed a GC procedure for quantitating homatropine methyl bromide based on the analysis of mandelic acid produced upon its hydrolysis. The first method does not distinguish between atropine and its main decomposition product, apoatropine, as the alcohol component of both compounds is tropine. A technique, based on the same principles as the second method[4], has now been developed for determination of the very small amount of atropine (0.05%) in Valeriana liquid extract.

Due to the polar characters of 5,5-disubstituted barbituric acids, their GC determination was accompanied by certain difficulties. However, since the introduction of derivatization procedures their GC analysis has been greatly simplified. In this work the phenobarbital, present in an amount forty-fold greater than that of atropine, has been methylated and analysed in the preparation —without previous extraction— by GC.

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EXPERIMENTAL

The preparation comprised atropine sulphate (0.0075 g), menthol (0.3 g), phenobarbital (0.45 g) and Valeriana liquid extract (total solid 10%, w/v, 14.24 g).

Instrumentation and procedure

A Carlo Erba Model GV gas chromatograph, equipped with a flame ionization detector, was used. The column was a glass tube (1.0 m × 3.0 mm I.D.) packed with 1.5% OV-101 on silanized Gas-Chrom P (100–120 mesh). Nitrogen was used as carrier gas at a flow-rate of 40 ml/min and the column was operated at 140°C for tropic acid and at 165°C for phenobarbital. Chromatograms were recorded on a Speedomax Model G recorder and integrated by a Carlo Erba Model 75 electronic integrator. The chart speed was 12.7 mm/min.

All reagents were of analytical grade and the drug substances used were of pharmacopoeial standard.

Analysis of atropine sulphate

The internal standard was a solution of 24 mg 2-naphthol in 10.00 ml of anhydrous diethylether.

To about 5.000 g of the preparation (equivalent to about 2.5 mg atropine sulphate) were added 10.0 ml of water. The solution was extracted with three 10-ml portions of chloroform and one 10-ml portion of anhydrous ether. Vigorous shaking was avoided. The chloroform and ether extracts along with any emulsion were discarded. The aqueous solution was acidified to pH 2 with 10% hydrochloric acid and the solution was extracted with three 10-ml portions of anhydrous ether. Vigorous shaking was again avoided. The ether extracts were discarded. The aqueous solution was adjusted to pH 13 with 10% sodium hydroxide solution and heated to boiling for 30 min. Following cooling, the solution was adjusted to pH 2.2 by using 10% hydrochloric acid and extracted with three 20-ml portions of anhydrous ether. To the combined filtered (through cotton–anhydrous sodium sulphate) extract was added 1.00 ml of 2-naphthol internal standard solution. The solution was evaporated to dryness on a water-bath. The residue was dried over silica gel and dissolved in 200 μl of N,O-bis(trimethylsilyl)acetamide (BSA) and the solution was allowed to stand at room temperature for 20 min. A 0.5-μl volume was injected into the GC column.

Analysis of phenobarbital

The methylating reagent, 0.05 *M* trimethylphenylammonium acetate solution, was prepared from N,N-dimethylaniline and methyl iodide according to Mráz and Šedivec[5]. The internal standard was a solution of 60 mg hexobarbital in 10.00 ml of 0.5 *M* trimethylphenylammonium acetate solution.

In a 10-ml volumetric flask about 1.000 g of the preparation (equivalent to about 3 mg of phenobarbital) was dissolved in 5.00 ml of hexobarbital internal standard solution and diluted to the mark with 0.5 *M* trimethylphenylammonium acetate solution. A 0.5-μl volume was injected into the GC column.

RESULTS AND DISCUSSION

Direct GC determination of atropine —without previous extraction— cannot be carried out due to the preponderant concentrations of various natural components in the preparation. Attempts to decrease the attenuation in order to increase the peak of atropine have not been successful. The usual extraction procedure for atropine base did not eliminate interfering co-extracted substances that elute at and near the retention time of atropine.

These inadequacies prompted us to investigate other GC routes. Applying the method of Grabowski *et al.*[4] to atropine seemed to be most promising. However, under the circumstances of the hydrolysis[4], atropic acid —besides tropic acid— was always formed and the hydrolysis was not complete. To avoid the formation of atropic acid the hydrolysis was tried at room temperature, on a water-bath, on a hot-plate and by varying the pH of the solution. The presence and amount of atropic acid as well as the extent of hydrolysis were checked by GC and paper chromatography[6].

Complete hydrolysis takes place in 30 min at pH 13 on a hot-plate. Degradation of atropine to apoatropine and/or tropic acid to atropic acid is negligible under these circumstances. The rate of hydrolysis of atropine is proportional to the hydroxide ion concentration and is enhanced by increased temperature[7]. The high rate of hydrolysis may prevent the formation of atropic acid in this case.

The extraction procedure described in the Experimental effectively separates atropine and tropic acid successively from all interfering substances. In Fig. 1 a chromatogram of tropic acid obtained from the preparation is shown.

Although the introduction of derivatization techniques removed the disadvantages of irreproducible adsorptions and tailings previously experienced in GC of 5,5-

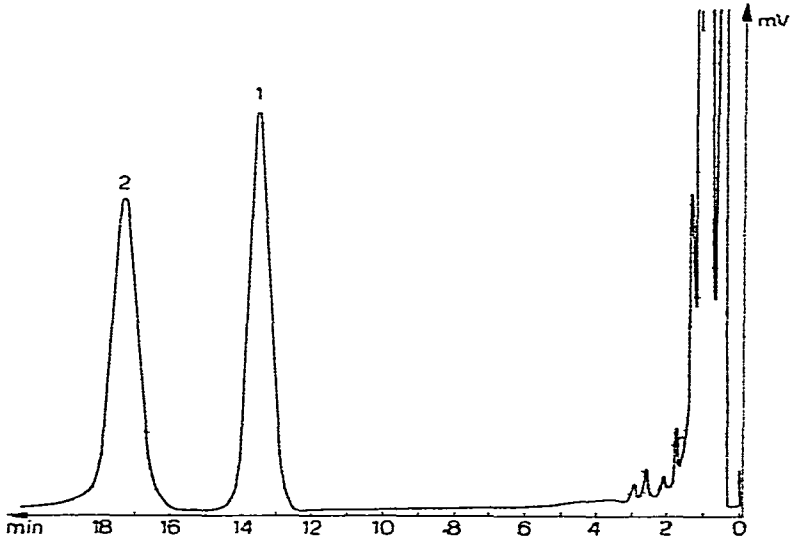

Fig. 1. Chromatogram of the preparation of silylated 2-naphthol (1) and silylated tropic acid (2). Column: 1.0 m × 3 mm. 1.5% OV-101 on Gas-Chrom P. Temperatures: injector 150°C, oven 140°C, detector 160°C. Nitrogen flow-rate: 40 ml/min.

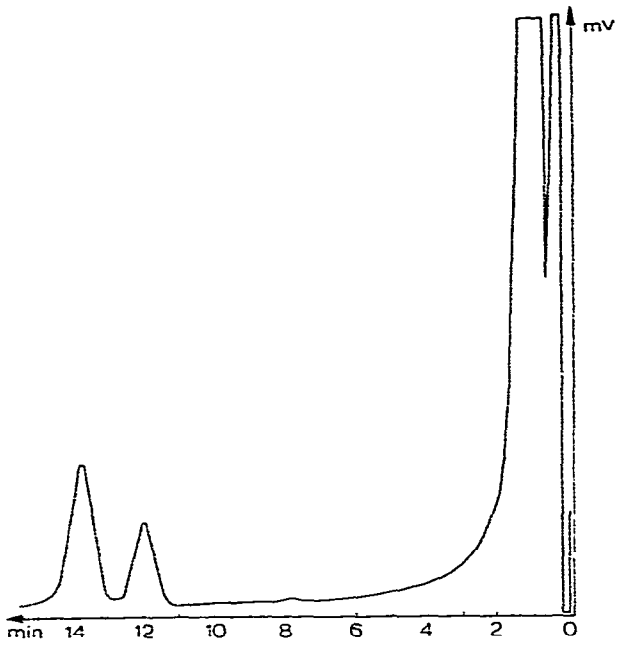

Fig. 2. Chromatogram of the preparation of methylated hexobarbital (1) and methylated phenobarbital (2). Temperatures: injector 180°C, oven 165°C, detector 200°C. Other conditions as in Fig. 1.

disubstituted barbituric acids, the composition of the derivatizing reagents remained a problem. Trimethylphenylammonium hydroxide, N,N,N-trimethylanilinium hydroxide and N,N-dimethylformamide (DMF) dialkylacetals[8,9] were often used as alkylating and acetal-forming agents. Methylation with the strongly basic quaternary ammonium bases produced cleavage of the barbiturate molecule[10] and/or formation of lower methylated products[11]. Impurities are very common in DMF and can interfere with peaks of barbiturates.

Mráz and Šdevic[5] proposed neutral reagents for methylation of barbiturates, the use of which produced the 1,3-dimethyl derivative of the corresponding barbituric acid without formation of by-products. This method has been applied with satisfactory results to methylate phenobarbital.

TABLE I

ACCURACY OF THE METHOD

Recovery experiments were carried out on laboratory-prepared mixtures of atropine and phenobarbital in 15.0 ml of ethanol. Results are means of the analysis of three samples, each of which was chromatographed six times.

Drug substance	*Weight (mg)*	*Found mean, $\bar{x}$ (mg)*	*Recovery, $\bar{x}$ (%)*
Atropine sulphate	7.50	7.35	98.0
Phenobarbital	450.00	448.00	99.0

TABLE II

PRECISION OF THE METHOD

The means, relative standard deviations and 95% confidence limits were determined by the analysis of three samples each of which was chromatographed six times.

Drug substance	*Label claim (mg)*	*Mean $\bar{x}$ (mg)*	*S_r (%)*	*95% Confidence limits (mg)*
Atropine sulphate	7.50	7.15	3.37	7.15 ± 0.12
Phenobarbital	450.00	441.00	1.53	441.00 ± 3.36

Methylated phenobarbital and methylated hexobarbital, the internal standard, are analysed directly in the dosage form. Previous extraction of phenobarbital is rendered unnecessary since there are no interfering peaks on the chromatogram of the preparation. Fig. 2 displays a chromatogram of hexobarbital and phenobarbital (both methylated).

The internal standrd method was used for quantitative evaluation. The ratio of the area of the peak of interest to the area of the internal standard peak was measured. Detector response factors relative to the internal standards were determined previously. Detector response linearities to tropic acid and phenobarbital were confirmed in the concentration ranges 0.5–2 mg/ml and 2–4 mg/ml, respectively.

Accuracy data for quantified components are given in Table I. Table II shows the precision data.

The procedure presented here for the assay of atropine and phenobarbital may provide an approach for their determination in other complex mixtures of pharmaceutical interest, too.

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