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Note

Determination of ambroxol in biological material by gas chromatography with electron-capture detection

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Ambroxol, a benzylamine derivative [*trans*-4-(2-amino-3,5-dibromobenzyl)-aminocyclohexanol hydrochloride], is a drug with mucokinetic properties that promotes alveolar surfactant synthesis [1,2]. Ambroxol in human plasma has been assayed radiochemically [3], by gas chromotography with electron-capture detection (GC–ECD) [4], and by high-performance liquid chromatography [5,6], after single or multiple oral or intravenous dosing. However, there are no reports of applications of these methods to samples of lung tissue and bronchial aspirate.

This paper describes a simple, selective and sensitive GC–ECD method for the determination of this compound in blood, lung tissue, bronchial aspirate and urine. Only small specimens of biological fluid or tissue are required because the derivatization with trifluoroacetic anhydride increases the ECD signal.

EXPERIMENTAL

Reagents and chemicals

Ambroxol ($C_{13}H_{17}Br_2N_2O \cdot HCl$) was supplied by Istituto De Angeli-Boehringer Ingelheim Italia (Milan, Italy) as Mucosolvan®. The internal standard, pinazepam, was supplied by Zambeletti Baranzate (Milan, Italy) and was used at a concentration of 5 ng/ml in methanol. Benzene methyl alcohol and glycine (analytical grade) were purchased from Farmitalia-Carlo Erba (Milan, Italy). *n*-Hexane and sodium hydroxide, analytical-reagent grade, were purchased from Merck (Darmstadt, F.R.G.). The buffer solutions for the partition study were

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made by mixing 2 *M* NaOH with 150 g of glycine (pH 11.0). Pure trifluoroacetic anhydride was purchased from Fluka (Buchs, Switzerland).

Source of biological materials

Blood and urine were collected according to standard procedures.

Tracheo-bronchial aspirate obtained from premature newborns with respiratory distress syndrome was supplied by the Department of Neonatal Pathology, University of Milan. These samples were obtained in the course of diagnostic and therapeutic investigations [7,8] by instillation of 0.5–1.0 ml of physiological saline solution into an endotracheal tube, followed by manual ventilation and aspiration. The pulmonary tissue specimens consisted of 1 g of healthy alveolar parenchyma obtained by a bioptic perimetral cone tissue excision. Adult human lung samples were obtained during surgery from patients with lung carcinoma, and were supplied by the IIIrd Chest Surgery Dept., Ospedale Policlinico, University of Milan.

Apparatus

GC analysis was done on a Dani 6500 instrument (Dani, Monza, Italy) with a capillary PTV injector equipped with an electron-capture detector, a Shimadzu C-R3A Chromatopac integrator, and a wall-coated open tubular (WCOT) fused-silica capillary column, (25 m × 0.25 mm I.D) coated with CP-Sil 5 CB, film thickness 1.2 μm (Chrompack, Milan, Italy).

The GC injector temperature was increased from 60 to 300°C, and the detector temperature was 290°C. The split was opened 42 s after injection of the total sample into the PTV injector, and the temperature programme was started. The column temperature was programmed from 240 to 260°C at 5°C/min. This temperature was held for 10 min, then raised to 280°C at 5°C/min, and this final temperature was held for 20 min in order to remove contaminants from the column. The carrier gas flow-rate (hydrogen through the capillary column) was 6 ml/min.

Procedure

Blood. Heparinized blood volumes ranging from 0.4 to 1 ml were placed in 30-ml vials. The specimens were mixed with redistilled water and diluted to 5 ml. Then 5 ml of 2 *M* glycine buffer (pH 11.0) were added. Ambroxol was extracted with 10 ml of benzene under horizontal shaking for 20 min. The samples were centrifuged at 2000 *g* for 15 min, and 9 ml of the benzene phase were recovered and evaporated to dryness on a Rotavapor. Then 5 ml of methanol together with 10 ml of *n*-hexane (as washing) were added to the dry residue, still under horizontal shaking. The samples were then centrifuged for 5 min at 2000 *g*, and the *n*-hexane phase was removed. The remaining phase, containing ambroxol, was dried on the rotating evaporator. The residue was redissolved in 0.8 ml of methanol and transferred to 1-ml vials. Excess solvent was removed by evaporation to dryness under a stream of nitrogen.

Lung tissue. Lung tissue (1 g) was homogenized in an all-glass Potter apparatus with 4 ml of 1.15% KCl. Then the samples were centrifuged at 12 000 *g* for 15 min. The top layer was diluted to 5 ml with redistilled water, and the extraction procedure described for blood was then followed.

Bronchial aspirate. Bronchial aspirate (2–5 ml) was centrifuged at 2000 *g* for 10 min, then placed in a 30-ml tube and diluted to 5 ml with redistilled water. The sample was then extracted as for blood.

Urine. Urine samples (2–5 ml) were centrifuged at 2000 *g* for 10 min, then extracted as for blood.

Derivatization

The organic phase was dried under a nitrogen flow, and 200 μl of trifluoroacetic anhydride were added. The vial was tightly capped and left to react for 35 min in a sand-bath at 90°C. The sample was then cooled in ice for 5 min, and evaporated to dryness under a nitrogen stream for 30 min. A suitable volume of the standard solution containing pinazepam (5 ng/ml) in methanol was added to the samples, and 1 μl of each was analysed chromatographically.

The molecular structure of the ambroxol derivative is shown in Fig. 1. Although pinazepam has only one halogen atom and the derivatized drug has eight halogen atoms, it was chosen as internal standard because its detector response is sufficiently sensitive.

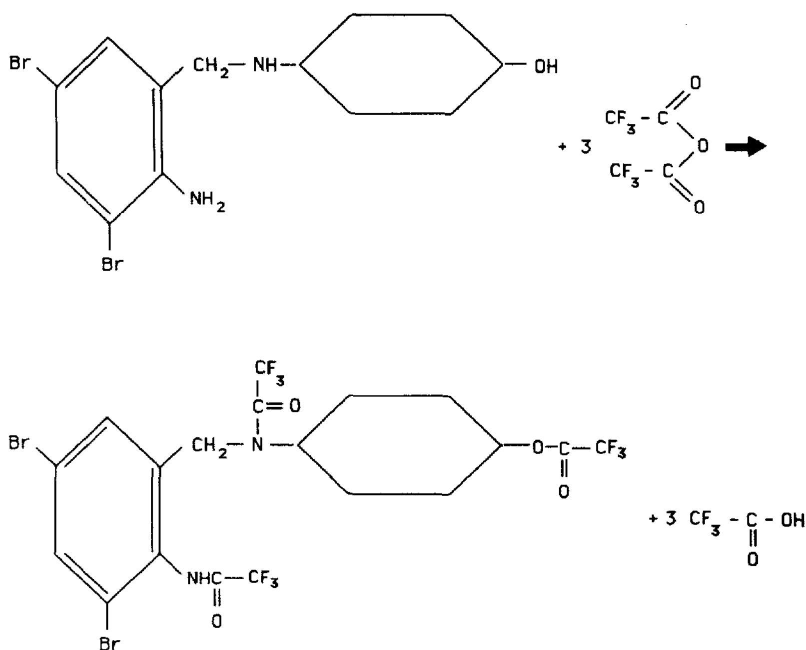

Fig. 1. Derivatization with trifluoroacetic anhydride.

Preparation of the calibration curve

The calibration curve was plotted using a solution of ambroxol in methanol at concentrations from 4 to 250 ng of ambroxol, placed in a 1-ml vial and dried under a nitrogen stream. The samples were derivatized as described above. A 1-ml volume of standard solution was then added to the final sample, and 1 μl was analysed by GC.

Recovery

For the recovery studies 150 ng/ml ambroxol was added to all samples of biological fluids: 1 ml of each material was then extracted and derivatized as described.

Stability

For the stability studies an amount of ambroxol corresponding to 150 ng per ml of material was added to a pool of bronchial aspirate fluid. The samples were left to incubate at room temperature for 30 min, which is the usual interval between drawing the sample and freezing it. The stability was checked at the following times by analysing 1 ml of each bilogical specimen: 0 and 30 min, and 1, 7, 14, 21 and 35 days after addition of ambroxol. Stability experiments were run in quadruplicate.

RESULTS AND DISCUSSION

The calibration curve of ambroxol indicates that there was a linear correlation between 4 and 250 pg of ambroxol and the ratio between the areas underneath the curves of ambroxol and pinazepam recorded by the electron-capture detector outside the GC column. The calculation of the linear least-squares regression ($y = a + bx$) resulted in a coefficient of correlation of 1.0.

Recovery was evaluated by analysing spikes of each kind of sample, and the result was 85 ± 1.7% S.D. The limit of detection was 4 ng/ml ambroxol for all the biological samples.

Because benzene, used for ambroxol extraction from biological samples, is carcinogenic, is may be replaced by toluene with slightly lower recovery.

Figs. 2 and 3 show the chromatograms for a blank blood sample, a standard solution of ambroxol (50 ng/ml), blood and lung tissue samples spiked with 50 ng/ml ambroxol. Chromatograms for urine and bronchial aspirate samples were similar to those reported for blood and lung samples.

The stability studies at 30 min and 1 day after the addition of ambroxol showed that the sample contained 100 ± 2.3% S.D. of the initial amount. On day 7 ($n = 4$) the content was 95 ± 1.8% and on day 21 it was 80 ± 1.2%. By day 35 the ambroxol content was 60 ± 0.9% of the initial amount.

A tailing peak was produced when ambroxol was analysed by GC because the presence of free hydroxyl and amino groups in this molecule results in column

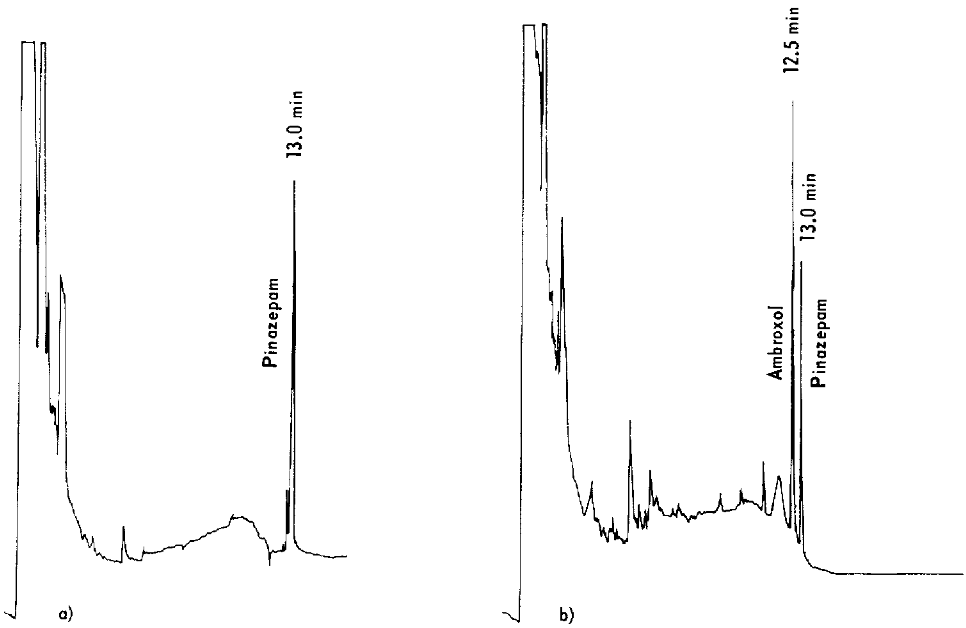

Fig. 2. (a) Chromatogram of a blank blood sample spiked with 5 ng/ml I.S. (b) Chromatogram of ambroxol standard solution (50 ng/ml) spiked with 5 ng/ml I.S.

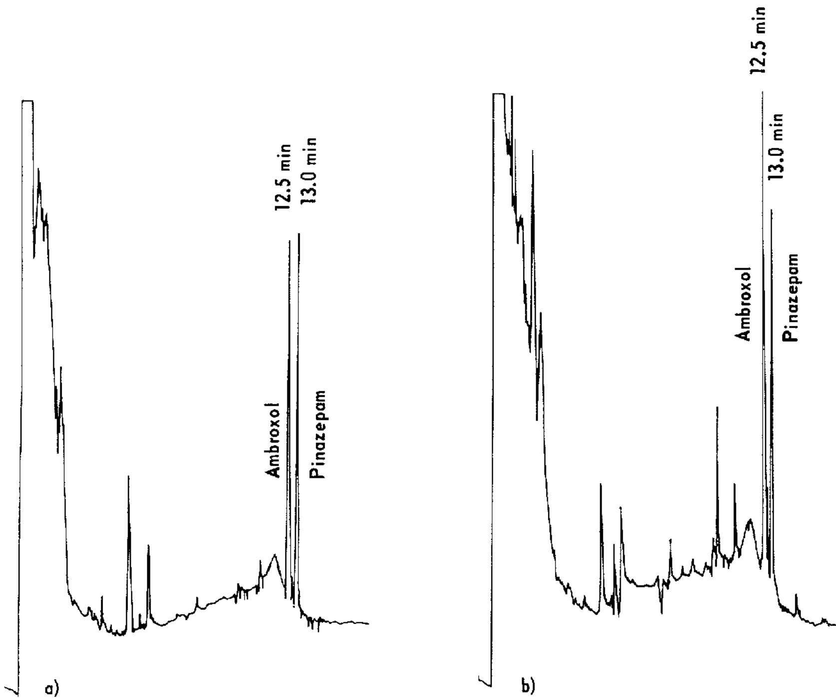

Fig. 3. Chromatograms of (a) lung and (b) blood samples containing 50 ng/ml ambroxol.

TABLE I

ISOTOPE CLUSTER ABUNDANCE CALCULATION OF TRIFLUOROACETYLAMBROXOL

The molecular formula is $C_{19}H_{15}N_2Br_2O_4F_9$. Carbon has two isotopes, hydrogen and oxygen three each, nitrogen and bromine two each, and fluorine one.

Mass	Abudance (%)
664	50.5594
665	11.3849
666	100.0000
667	22.3257
668	51.0384
669	11.1166
670	1.5760
671	0.1684
672	0.0145
673	0.0010

absorption. Quantification is poor because of a non-linear, non-reproducible response. It was therefore decided to form a trifluoroacetyl derivative of ambroxol to improve its GC properties. Trifluoroacetylation was preferred to silylation so as to avoid the formation of a cyclic compound, a quinazoline derivate. The synthesis of trifluoroacetylambroxol was confirmed by GC–MS (Table I and Fig. 4) with standard samples of ambroxol in methanol. The mass spectrum of the ambroxol derivative indicates that all three reactive sites (hydroxyl, primary and secondary amino groups) were blocked by the trifluoroacetyl group.

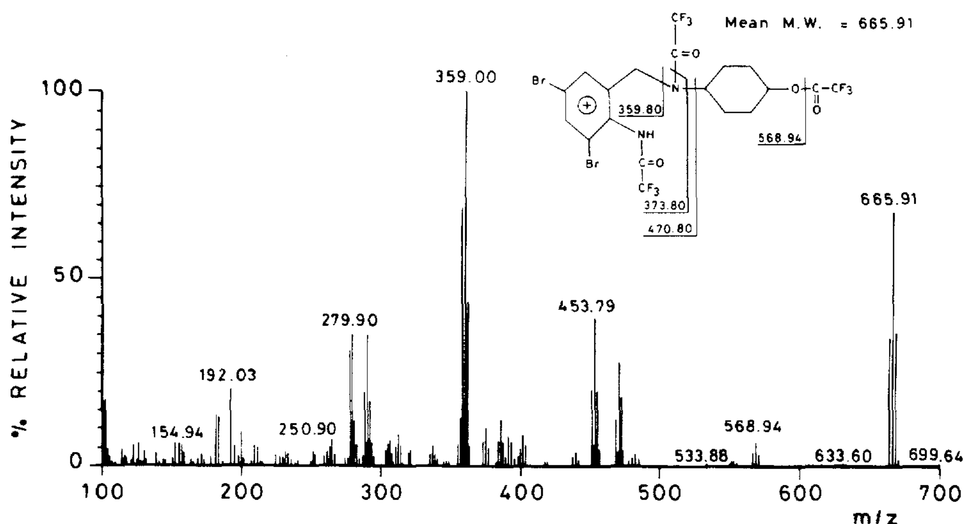

Fig. 4. Mass fragmentation pattern of trifluoroacetylambroxol.

Application

This GC assay of ambroxol was done on whole blood because preliminary data indicated that the drug splits between plasma and red cells. This method was employed in a pharmacokinetic study of ambroxol in the preterm newborn as it only requires very small samples for analysis [7]. The method proved particularly useful for pharmacokinetic studies of ambroxol in human chest surgery. The results, which indicate higher concentrations in lung tissue than in blood, will be reported elsewhere. To our knowledge ambroxol levels in lung tissue have not been reported or measured before.

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