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RADIOACTIVE LABELLING OF UNSATURATED COMPOUNDS WITH ^{125}I AND ^{131}I FOR ANALYSIS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

It is demonstrated that unsaturated compounds can be determined by iodination procedures with radioactive iodine. Fatty acids and barbiturates with unsaturated side-chains were used as model systems. The detection limits obtained with this procedure at a signal-to-noise ratio of 3:1 are 20 ng of butalbital per spot using ^{125}I and 5 ng per spot with ^{131}I. The reproducibility of these techniques is *ca.* 2% (relative standard deviation) for concentrations greater than 0.5 μg per spot and *ca.* 4% or higher for amounts less than 0.1 μg per spot.

The method is generally applicable to a number of unsaturated compounds; others, such as ergotamine and codeine, exhibit some uncontrollable secondary reactions and therefore cannot be measured. In general, the reaction is additive, *i.e.*, the number of double bonds determines the sensitivity of the method.

INTRODUCTION

During our investigations for new derivatization procedures that would cover all possible structural types, no technique has been devised that can be used for double bonds in unsaturated compounds. This would have been of particular interest for compounds with otherwise poor detection properties.

The addition of halogens can be adapted as a general method for the determination of unsaturated compounds. The technique has been discussed for the reaction of bromine[1], iodine bromide[2] and iodine chloride[3] with fats and fatty acids and for bromometric titrations of barbiturates with unsaturated side-chains[4]. A radiometric procedure with ^{131}IBr in methanol in conjunction with paper chromatography has been discussed by Kaufmann and Budwig[5].

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The purpose of this work was to investigate the possibility of using radioactive iodine (^{125}I and ^{131}I) for this purpose. Compounds that can be iodinated in a quantitative and reproducible reaction should then be amenable to direct measurement with a radioscanner following thin-layer chromatographic (TLC) separation.

EXPERIMENTAL

Apparatus

Berthold Model II LB 2723 TLC radioscanner (Berthold, Karlsruhe, G.F.R.) equipped with a rate meter, an integrator and a Philips Model PM 8221 dual-pen recorder (Philips, Eindhoven, The Netherlands) and a Zeiss Chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) were employed.

Reagents and materials

Methanol, chloroform, carbon tetrachloride, glacial acetic acid, diethyl ether and iodine bromide (p.a. grade) were obtained from Merck (Darmstadt, G.F.R.). $Na^{125}I$ and $Na^{131}I$ were obtained from the Eidgenössische Institut für Reaktorforschung, Würenlingen, Switzerland.

Silica gel 60 F_{254} TLC plates (Merck) were pre-washed twice with methanol–chloroform (3:1). Extrelut® columns were obtained from Merck.

Test substances

Butalbital and allobarbital were supplied by Sandoz (Basle, Switzerland), secobarbital by Bertalanffy (Unterach, Austria) and aprobarbital by Hoffmann-La Roche (Basle, Switzerland).

Oleic acid, linoleic acid, linolenic acid and triolein (all puriss.) were purchased from Fluka (Buchs, Switzerland). Linseed oil and olive oil (DAB IX) were purchased from Herba (Graz, Austria).

Standard solutions of the substances were prepared in chloroform.

Preparation of the radioactive IBr solutions

An amount of $Na^{125}I$ or $Na^{131}I$ corresponding to 10–200 μCi was transferred into a conical ampoule and evaporated to dryness in a vacuum desiccator over calcium chloride. The residue was dissolved in 100 ml of a 0.01–0.1 *M* solution of IBr in a carbon tetrachloride–acetic acid (5:1). The IBr can be purchased commercially or prepared in the laboratory by mixing equimolar solutions of iodine and bromine. The reagent must be prepared freshly every day.

General reaction procedures

In vitro. The sample solution was evaporated to dryness in a 0.5-ml conical vial with a screw-cap and a PTFE seal. A 10-μl volume of reagent containing at least a 5-fold excess per reactive group (0.05–0.1 *M* IBr solution with an activity of 10–50 μCi per 100 μl for amounts greater than 5 nmoles; 0.01–0.02 *M* IBr solution with an activity of 50–200 μCi per 100 μl for amounts less than 5 nmoles) were added to the residue. The reaction vial was stoppered and left to react for 30 min in a refrigerator. A 5-μl volume of the mixture was taken from the vial with a syringe and spotted on a TLC plate. Eight spots can be placed on a 20-cm plate. The plate was placed at room temperature for 1 h in a vacuum desiccator over potassium hydroxide to

remove excess of reagent. This procedure was followed by the chromatographic development.

In situ. A 2-μl volume of the sample in chloroform was spotted with microcaps on the TLC plate. After drying with an air stream and cooling the plate in a refrigerator, 5 μl of reagent were applied on top of the sample spot and the plate was stored in the dark for 30 min. After drying in a vacuum desiccator for 1 h the plate was developed.

Chromatography

The solvent system for unsaturated fatty acids was chloroform–methanol (95:5, v/v), while for the barbiturates chloroform–acetic acid (95:5, v/v) was used. R_F values are given in Table I.

TABLE I

R_F VALUES FOR FATTY ACIDS, FATS AND BARBITURATE

Type	*Compound*	*IBr derivative*	*Parent compound*
Fatty acids	Oleic acid Linoleic acid Linolenic acid	0.40	0.45
Fats	Triolein Linseed oil Olive oil	0.80	0.85
Barbiturates	Butabital	0.37	0.50
	Allobarbital	0.27	0.45
	Secobarbital	0.43	0.54
	Aprobarbital	0.30	0.42

The chromatograms were developed for 15 cm after conditioning of the chromatographic chamber for 1 h. Prior to measurement the plates were dried in an air stream.

Measurement conditions

The counting gas for the gas flow proportional counter (voltage 1700 V) was P_{10} (methane + 10% argon). The focusing array was 16 mm long and 2 mm wide, without a window.

Conditions for amounts greater than 5 nmoles. The following conditions were used: scan rate, 300 mm/h; count rate, 10,000–20,000 cpm; time constant, 10 sec; integrator, set for 1000–2000 counts; speed of recorder paper, 2 cm/min.

Conditions for amounts less than 5 nmoles. The following conditions were used: scan rate, 120–300 mm/h; count rate, 2000–10,000 cpm; time constant, 50 sec; integrator, set for 1000–2000 counts; speed of recorder paper, 2 cm/min–18 cm/h.

The peak areas were evaluated either by calculating the height $\times$ peak width at half-height or by electronic integration.

Procedure for the determination of barbiturates in blood and plasma samples

Samples of 4 ml of whole blood or plasma stabilized with citrate and containing

0.5–100 μg of barbiturate were diluted with 15 ml of distilled water and 1 ml 1 *N* hydrochloric acid, giving a pH of 3. The total sample solution was applied to an Extrelut column[6], left for 20 min and then eluted with 50 ml of diethyl ether. The eluate was filtered over anhydrous sodium sulphate and evaporated to dryness in a conical ampoule over nitrogen. The residue was dissolved in 20–100 μl of chloroform and an aliquot of the solution was treated as described previously.

The presence of unsaturated lipids in the plasma samples demanded the use of a large excess of reagent, with a corresponding higher activity.

For quantitative work one or several standards were treated simultaneously by the same procedure.

Procedure for tablets

One tablet was pulverized and extracted three times with 15-ml portions of methanol containing 2% (w/v) of tartaric acid. The combined extracts were diluted to 50 ml with methanol. An aliquot was then treated as described above.

RESULTS AND DISCUSSION

The optimization tests for completeness and reproducibility of the iodination step were carried out with non-radioactive IBr solutions. At higher concentrations quantitative measurements could be carried out by densitometric scanning. The scans

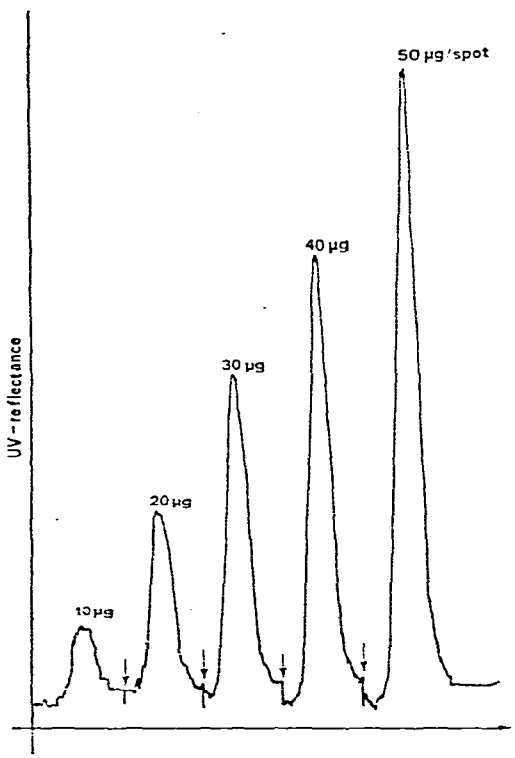

Fig. 1. Densitometric scan for different concentrations of iodinated oleic acid developed on a silica gel thin-layer chromatogram. Scan rate, 100 cm/h; recorder, 60 cm/h; detection wavelength, 218 nm.

for a test series of 10–50 μg of oleic acid per spot are shown in Fig. 1. The correlation coefficient was 0.998. Similar results were found for the barbiturates.

It can be recommended that the method be optimized by this technique for other groups of unsaturated compounds.

Reaction with ^{125}IBr (half-life of ^{125}I = 60 days)

The reproducibility was tested for different concentration ranges of butalbital, and was found to be about 2% (relative standard deviation for amounts greater than 0.5 μg per spot (n = 8). For amounts to about 0.1 μg per spot a relative standard deviation of 4% or higher was observed.

This is easily explainable as the experimental conditions lie close to the detection limit of about 20 ng per spot at a signal-to-noise ratio of 3:1 (see Fig. 2).

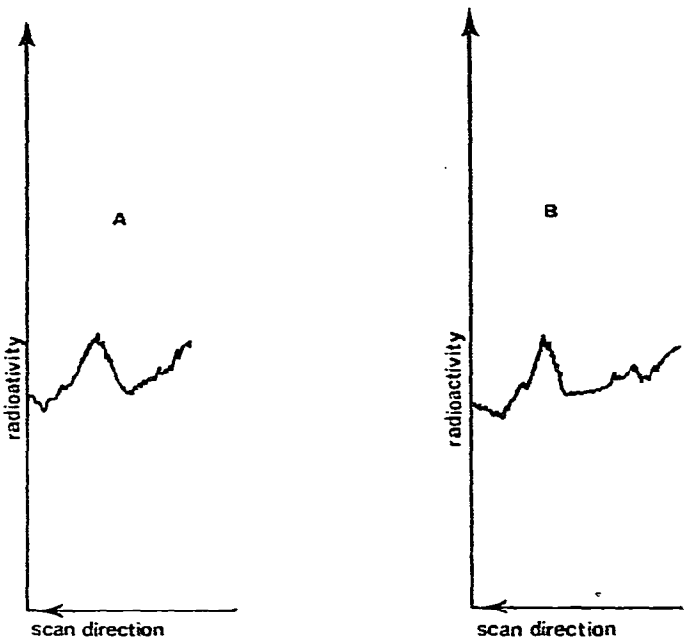

Fig. 2. Comparison of scans for 50 ng of butalbital per spot, reacted with ^{125}IBr and developed on a silica gel plate. (A) Reaction *in situ*; (B) reaction *in vitro*. 5000 cpm; 50 sec; 120 mm/h; Rec. 18 cm/h (these abbreviations of the measurement conditions here and in subsequent legends mean the following: A count rate of 5000 cpm gives a full-scale response of the recorder. The time constant of the recorder amplifier is adjusted in such a way that it takes 50 sec to reach the full-scale deflection. 120 mm/h is the scan rate for the TLC plate. 18 cm/min is the recorder chart speed).

For counting of the ^{125}I, the detection limit can be decreased by a factor of 10 by using a sodium iodide scintillation counter, but this was not available at the time of these studies.

The linearity of calibration graphs was tested for the ranges 1–10 and 0.1–1.0 μg per spot (see Figs. 3 and 4). The correlation coefficient was 0.9985 for both ranges. The possibility of carrying out the reactions directly on the plates (*in situ*) was also investigated (see also Experimental), the procedure thereby being simplified. The reproducibility was comparable to that of *in vitro* techniques.

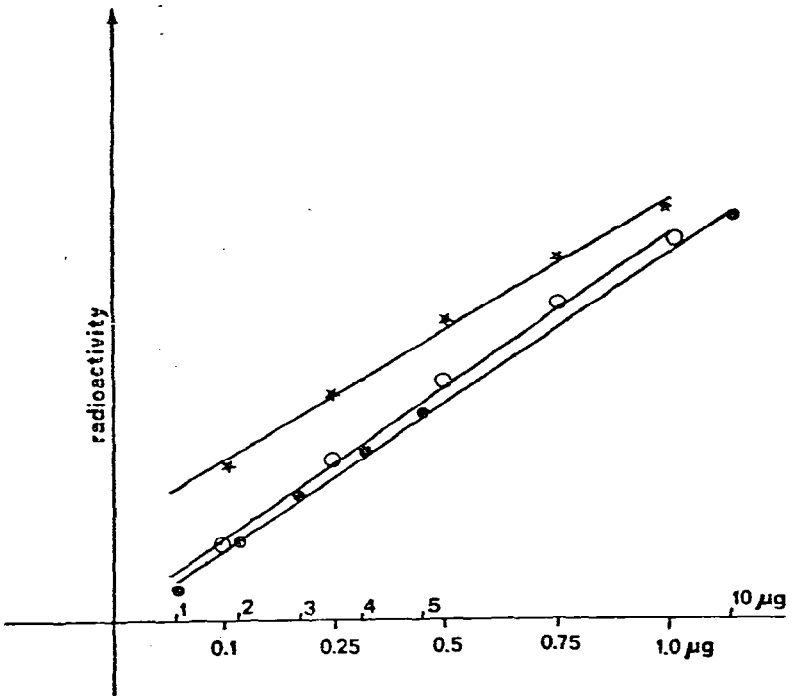

Fig. 3. Calibration graphs for various concentrations of butalbital, reacted with ^{125}IBr and developed on a silica gel thin-layer chromatogram. ★, Concentration range 0.1–1 µg per spot, reaction *in situ*; ○, 0.1–1 µg per spot, reaction *in vitro*; ●, 1–10 µg per spot, reaction *in vitro*.

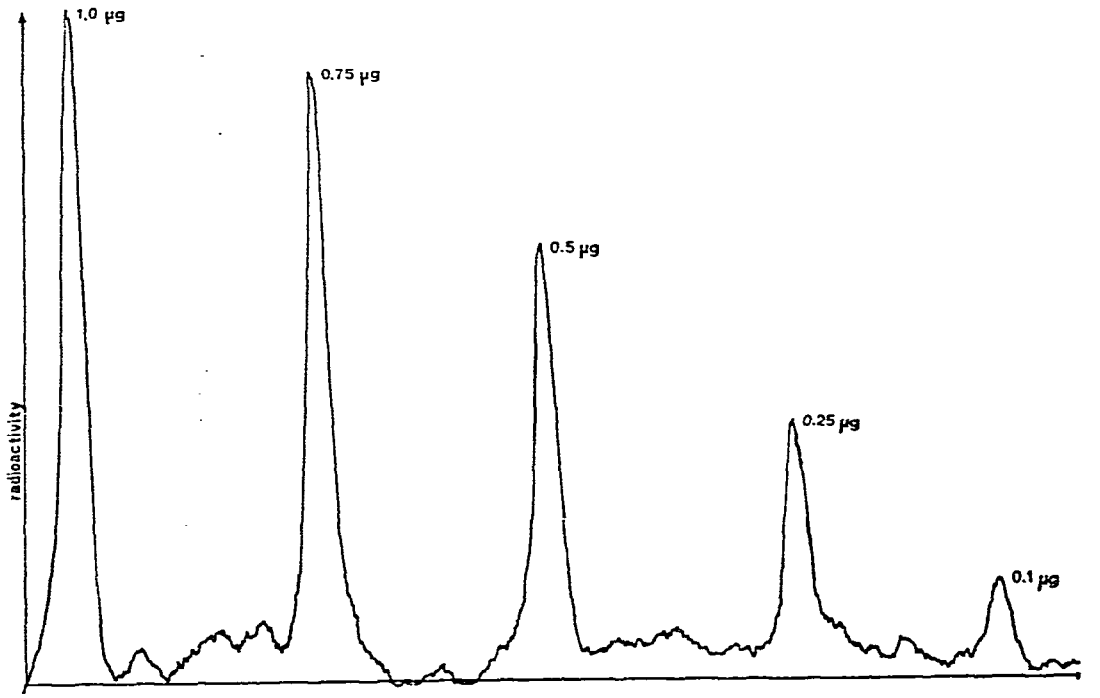

Fig. 4. Scans for various concentrations of butalbital in the concentration range 0.1–1 µg per spot. Reaction (*in situ*) directly on the plate. 10,000 cpm; 50 sec; 120 mm/h; Rec. 18 cm/h.

Reaction with ^{131}IBr (half-life of ^{131}I = 8 days)

By iodination with ^{131}IBr it is possible to decrease the detection limit for butalbital to 5 ng per spot (signal-to-noise ratio 3:1; see Fig. 5). An investigation of the calibration graphs in the concentration range 10–75 ng per spot showed a satisfactory correlation (Fig. 5). The disadvantages, however, are also obvious: a shorter half-life and stronger radiation render the handling of ^{131}I more difficult; interferences from the solvent are also more pronounced and result in baseline problems.

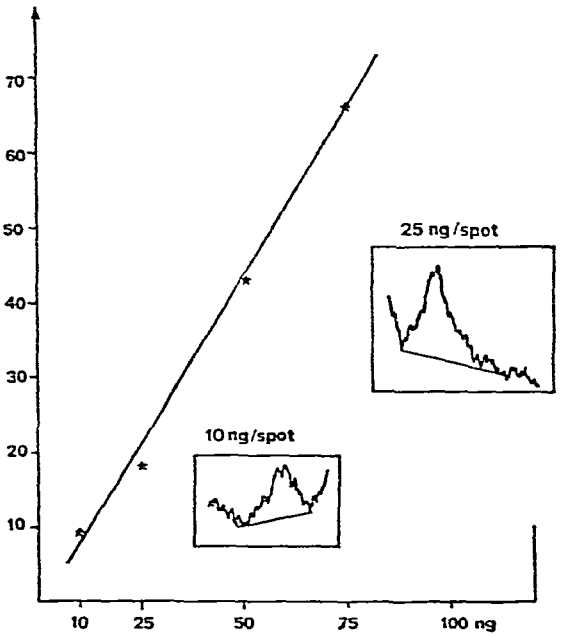

Fig. 5. Calibration graph and scans for various concentrations of butalbital, reacted with ^{131}IBr. Concentration range 10–75 ng per spot; reaction *in situ* with 0.02 *M* ^{131}IBr. 5000 cpm; 50 sec; 120 mm/h; Rec. 18 cm/h.

Study of other compounds and application

As already shown before, oleic acid (and other fatty acids) can be handled successfully by this technique. The study of other barbiturates with double bonds is also feasible. Iodination of allobarbital, which contains two double bonds, resulted in exactly twice the activity and a correspondingly lower detection limit. Linoleic acid (two double bonds) exhibited twice the activity and linolenic acid (three double bonds) three times the activity of oleic acid, which has only one double bond (see Fig. 6).

The reactions with codeine or with ergotamine did not yield uniform products; here competitive substitution or oxidation reactions are likely to interfere.

Application to blood and plasma samples

The method was applied successfully to whole blood and plasma samples with concentrations as low as 0.5 μg/ml of barbiturate. The clean-up with Extrelut columns combined with the subsequent chromatographic separation makes the

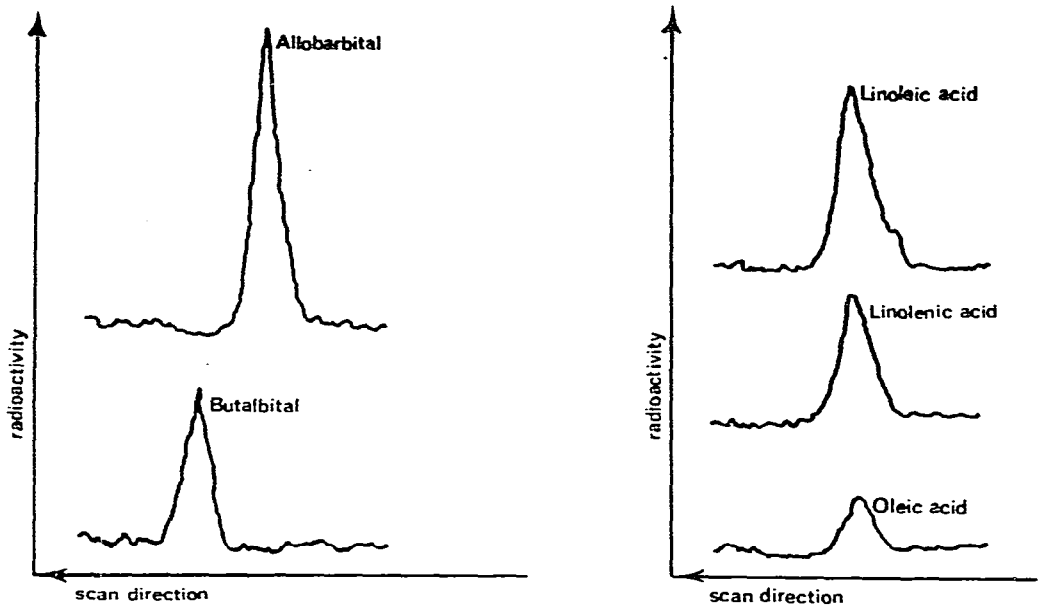

Fig. 6. Left: comparison of scans for equal amounts of butalbital and allobarbital (0.5 μg per spot; reaction *in situ* with 0.02 *M* ^{125}IBr). Right: comparison of scans for equal amounts of oleic, linoleic and linolenic acid (0.5 μg per spot; reaction *in situ* with 0.02 *M* ^{125}IBr). 20,000 cpm; 10 sec; 300 mm/h; Rec. 12 cm/min.

method extremely specific. Interferences by iodinated proteins or plasma lipids do not occur under the conditions used.

A typical chromatogram for the determination of butalbital in whole blood samples of rabbits is shown in Fig. 7. Samples were taken from the ear 30 min after intraperitoneal injection of 30 mg/kg. The amount in Fig. 7 corresponds to *ca.* 1.2 μg of butalbital in the 4-ml sample. The recovery of butalbital (5 μg/ml) from plasma samples was 82% ($n = 8$).

The reproducibility of the measurements ($n = 8$) is approximately 3% relative standard deviation for 50 μg/ml, 7% for 5 μg/ml and 15% for 0.5 μg/ml.

Application to a pharmaceutical formulation

Tablets of Plexonal forte® (composition: butalbital sodium 75 mg, phenobarbital sodium 45 mg, barbital sodium 135 mg, dihydroergotamine methanesulphonate 0.48 mg and scopolamine hydrochloride 0.24 mg) were analysed according to the precedure described under Experimental. The method permitted the selective assay of the unsaturated barbiturates in the presence of the other active principles. The values were within the tolerance limits, *i.e.*, 74.6 mg of butalbital per tablet. A relative standard deviation of 2.3% was observed for eight successive measurements on the same tablet.

Determination of iodine numbers on the micro-scale

The degree of unsaturation in fats and oils at the microgram level can conveniently be measured by this technique. In addition, its application to the determi-

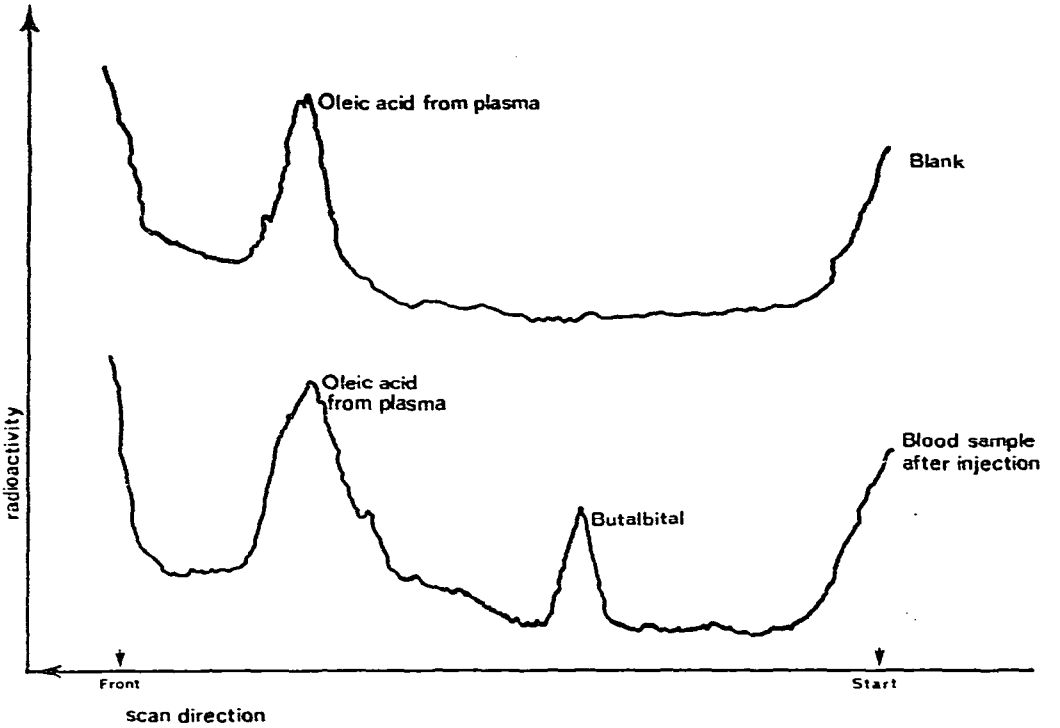

Fig. 7. Determination of butalbital in rabbit blood samples. Concentration, 1.2 μg per 4 ml. Reaction *in situ* with 0.05 *M* ^{125}IBr. 20,000 cpm; 10 sec; 300 mm/h; Rec. 2 cm/min.

nation of free unsaturated fatty acids and mono-, di- and triglycerides was investigated following chromatographic separation. A known amount of oleic acid (theoretical iodine number = 90) was added to the sample as an internal standard.

CONCLUSION

It has been demonstrated that certain unsaturated compounds can be determined quantitatively and with good sensitivity by iodination with radioactive iodine at the double bond. This should be generally valid for many groups of compounds containing C = C double bonds and is particularly useful for compounds that are difficult to detect otherwise (*i.e.*, pheromones). The low detection limits (low nanogram or even picogram level) and the selectivity of this technique should render it useful for trace investigations in complex matrices.

Replacing the gas flow proportional counter with a sodium iodide scintiliation counter should result in an about a ten-fold decrease in the detection limit. Some application possibilities are currently under scrutiny. The adaptability of transfer of this technique to high-performance liquid chromatography is also being investigated. The additivity aspect demonstrated with allobarbital should be interesting for standardization when analysing mixtures of such compounds. It should be mentioned however, that the method can be performed only in laboratories with special equipment for work with volatile radioactive isotopes.

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